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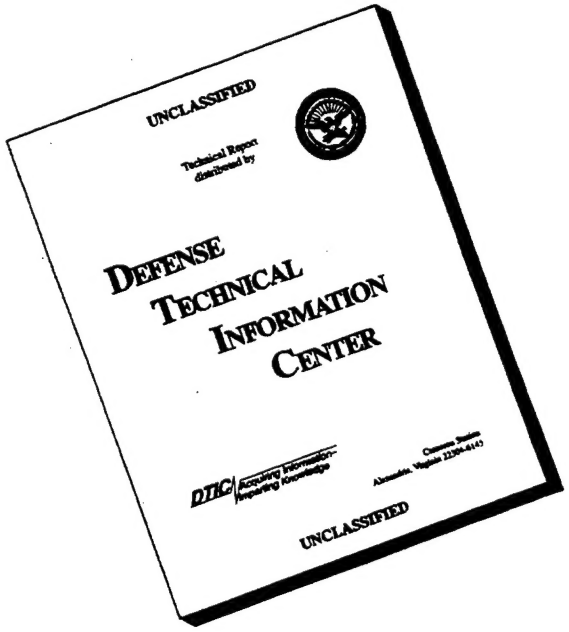

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13. ABSTRACT (Maximum 200 words)

Certain inhaled toxins, e.g., phosgene and other oxidant gases, can trigger a debilitating and usually fatal form of respiratory distress. Presently, there is no antidote for such lung-damaging agents. We have completed a comprehensive examination of surfactant replacement therapy (SRT) as a counter-measure against these agents which damage the pulmonary surfactant system of the alveolar and respiratory bronchiolar airways. Exogenous pulmonary surfactant was administered either by intratracheal instillation or by aerosol to male Sprague-Dawley rats that had been exposed to 40.5 ppm phosgene gas for 10 minutes in a Cannon type (nose-only) chamber [the LCt_{50}, 24-hour] in order to assess treatment effects on tissue edema, lung function and survival. Phosgene exposure was found to have an adverse effect on the surface activity of the endogenous pulmonary surfactant system in these exposed rats. Surfactant replacement helped to restore this activity. SRT did not prevent massive outpouring of edema water which marks the clinical phase of phosgene poisoning, nor did it alleviate the attending decline in lung function. Nonetheless, SRT was found to significantly reduce mortality from exposure to the toxin. Our findings suggest that SRT may be the first effective countermeasure for US military personnel following lethal phosgene exposure.

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William D Currie 10/13/95

PI - Signature Date

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Introduction

Phosgene is an extremely reactive molecule that nonspecifically and irreversibly acylates macromolecules in the lung parenchyma [1]. It is a potent pulmonary toxin and one of several CW agents that can trigger a debilitating and usually fatal form of respiratory distress. There are at present no antidotes for phosgene poisoning and few if any effective countermeasures [2].

In this report, we will summarize the results of studies examining the effectiveness of surfactant replacement therapy (SRT) as a countermeasure for phosgene poisoning. SRT is being used to correct deficiencies in the lung's native pulmonary surfactant (PS). SRT is now a standard procedure in the treatment of neonatal respiratory distress syndrome and may soon become one in the treatment of adult respiratory distress syndrome [3-5]. Both laboratory and clinical findings suggest that SRT is an effective treatment for restoring and maintaining the alveolar surface film under conditions in which the endogenous PS system is either deficient or damaged [6-11].

Surfactant is an essential component of the alveolar lining fluid (ALF) [12,13]. It is secreted by type II pneumocytes into the ALF where it serves to lower the surface tension force at the air-liquid interface of the alveolus and, as a consequence, to maintain alveolar structural stability, to decrease the work of breathing and to prevent alveolar flooding [12-14]. There are several mechanisms by which phosgene inhalation adversely affects these PS functions, including direct acylation of PS constituents and/or enzymes involved in PS metabolism and clearance [15,16]. Phosgene increases pulmonary microvascular permeability to proteins and causes circulating PMN leukocytes to enter the lung tissue and alveoli [17,18]. Some of the serum proteins which leak into the ALF and admix with the PS, e.g., fibrin monomer, profoundly inhibit PS surface activity [19-21]. Inflammatory cells infiltrating the alveoli release proteases and oxygen metabolites, which have been shown to adversely affect PS function [22-24].

Pattle originally proposed that by lowering surface tension in the alveoli, the PS acted to oppose transudation of fluid from the capillaries and thereby prevented alveolar flooding [12]. According to this concept, the clinical rationale for using SRT to restore or maintain the alveolar

surface film in phosgene-exposed lungs would be to prevent edema, the most significant clinical manifestation of lethal exposures to phosgene gas. Secondary benefits would include improved lung compliance [12-14]. The only prior work on the effects of SRT in phosgene poisoning was performed several years ago by Scarpelli and his colleagues [25]. These investigators showed that SRT helps to normalize lung compliance in mongrel dogs following exposure to 94 ppm phosgene for 20 minutes. However, these investigators did not examine the effects of SRT on pulmonary edema or mortality.

This project represents the first comprehensive analysis of the effects of SRT on phosgene-poisoned lungs. Using adult Sprague-Dawley rats exposed to an $LCt_{50,10,24}$ dosage (10 minute exposure, mortality determined at 24 hours), we have confirmed that phosgene inhalation adversely affects the surface activity of the PS system and we have shown that therapeutic dosages of an exogenous surfactant can be delivered to the lungs by either direct instillation or as an aerosol to restore and maintain endogenous pulmonary surfactant function.

SRT appears to have little if any effect on two of the most prominent, pathophysiological manifestations of phosgene exposure: pulmonary edema and lung stiffness. Nonetheless, SRT is an effective countermeasure against the lethal effects of high dose phosgene exposure. SRT instituted up to 6 hours after exposure to a lethal dose of phosgene will significantly reduce the likelihood of death from the exposure.

Two of the surfactant replacements used in our studies are commercially available: EXOSURF, a completely synthetic surfactant, and INFASURF, a calf lung lavage extract of pulmonary surfactant. Thus, technology is available for treating military personnel should the effects of SRT in man parallel those observed in the phosgene exposed rat. Our studies clearly indicate the potential of SRT as a countermeasure for lethal phosgene exposure.

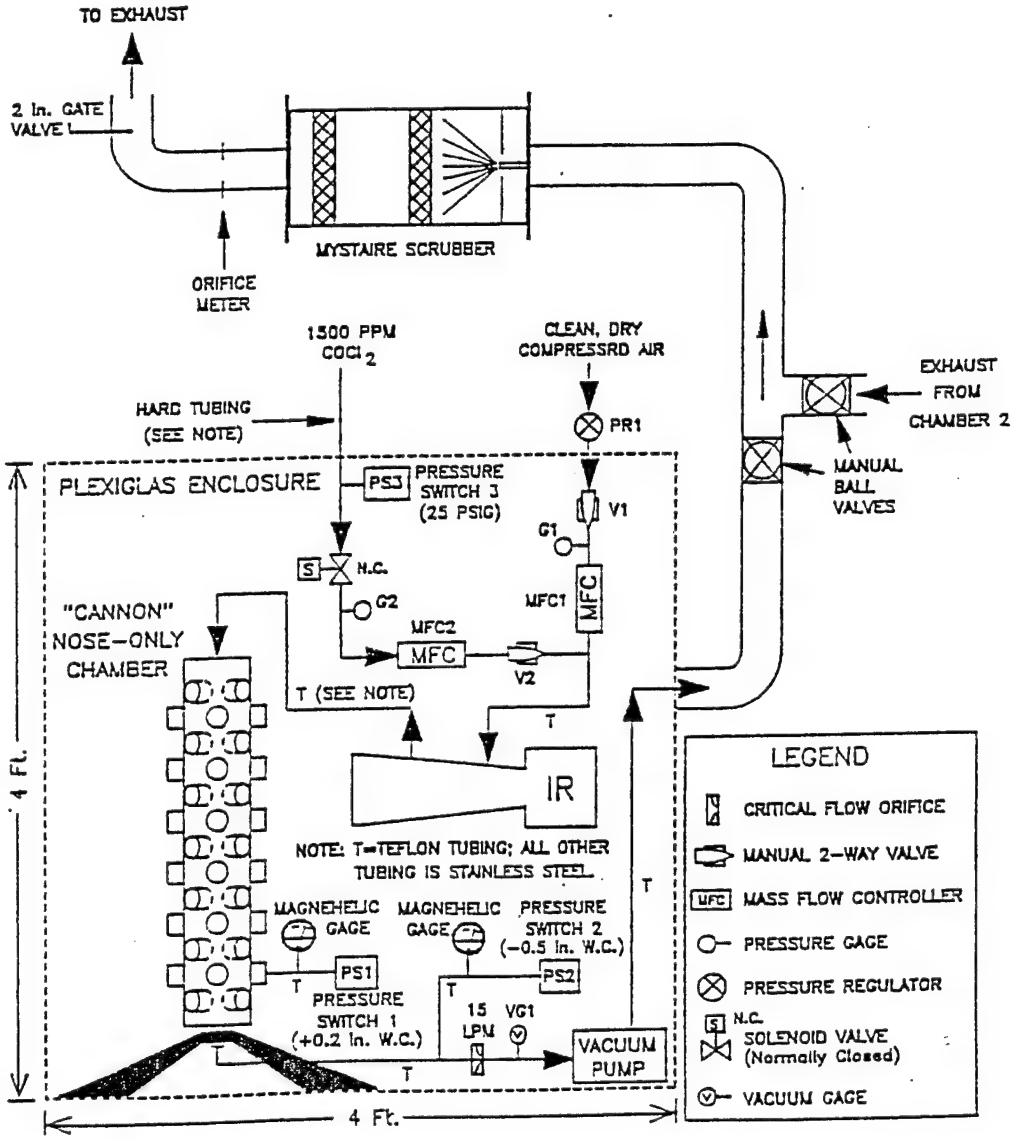
Methods

Exposures

All phosgene exposures were performed using a Cannon-type, nose-only exposure chamber (Lab Products, Inc.; Maywood, NJ), which was housed in the Air Toxics Laboratory of the US Environmental Protection Agency, Research Triangle Park, North Carolina. The exposure chamber consisted of a 52-position, nose-only tower with a 1-liter internal volume, through which phosgene gas was delivered to restrained male, Sprague-Dawley rats for 10 minutes in concentrations of up to 90 ppm. Phosgene was continuously delivered at a flow rate of 20 L/minute into the central chamber of the exposure apparatus from the top, flowing downward through the exposure tower and exiting from the nose ports to each animal. The exhaust was drawn away from the animals back into the tower, exiting through the bottom of the chamber to a Mystaire water scrubber before release to a common lab plenum (see Figure 1).

The phosgene concentration delivered to the animals was regulated by mixing a stream of concentrated phosgene gas from an outdoor cylinder with a stream of dry air (19 L/minute). The range of phosgene doses used in these studies could be attained by adjusting the flow of concentrated phosgene gas (1765 ppm in nitrogen) to between 0 and 1 L/minute using a Tylan mass flow controller (Tylan Inc., Torrence, CA). Phosgene concentration delivered to the animals was determined from the measured phosgene concentration in the outside cylinder and the respective flow rates of concentrated phosgene gas and dry air. The analytical concentration of phosgene in the cylinder was determined using a dynamic gas-blending system to reduce the phosgene concentration to below 1 ppm, and then quantified by gas chromatography using an electron capture detector calibrated against a certified (±2%) permeation phosgene tube. Total uncertainty in the estimate of phosgene concentration delivered to the animals was 5%. Phosgene concentration in the airstream delivered to the chamber was continuously monitored with an in-line infrared gas analyzer (Foxboro Miran 1A IR). The concentration of phosgene delivered to the chamber never exceeded the desired concentration by more than 10%.

Figure 1
Phosgene Exposure System



The entire exposure and monitoring system was enclosed in a custom-designed fume hood to prevent contamination of the lab if phosgene leaked from the primary containment chambers. All exposure personnel were supplied with Scott SKA-PAK full face masks with self-contained air for emergency use in case phosgene leaked beyond this secondary containment barrier. A dedicated IR capable of detecting phosgene concentration of 0.06 ppm (below the TLV phosgene of 0.1 ppm) was used to continuously monitor for ambient phosgene levels in the laboratory throughout the exposure.

Protocols

LCt_{50} determination

Animals were exposed in groups of 10 to one of five different doses of phosgene (200 to 700 ppm·min, 10-minute exposures). Following exposure, animals were removed from the exposure chamber tubes and lightly anesthetized with halothane. The trachea was subsequently intubated perorally with an 18-gauge cannula, and a 0.5-ml bolus of 0.9% NaCl in sterile water was instilled directly into the airway after positioning the tip of the cannula 1 cm above the tracheal bifurcation. Twelve of the rats died during or immediately after instilling the saline (see Table 1). These animals were not included in determining the LCt_{50} dosage. Animals which recovered from the halothane were returned to cages and provided with water and food ad libitum. All survivors were sacrificed at 24 hours after exposure.

Treatment effects

Sets of 16 to 20 animals were exposed at the same time to an $LCt_{50,10,24}$ dosage of phosgene. Exposure cohorts were divided into four treatment groups. One of the groups was used as a control. The animals in this control group received no surfactant replacement but were otherwise handled identically to their surfactant-treated, exposure cohorts. Animals were lightly anesthetized with halothane for instillation of the surfactant replacement. Animals which successfully recovered from the anesthetic were returned to cages and given water and food ad libitum until sacrifice. Animals which did not recover from the anesthetic were not used in assessing treatment effects. The numbers of rats killed by the halothane and/or instillation and the

Table 1: **LCt_{50} determination.** Comparison of number of animals exposed and number of animals tolerating halothane and saline instillation immediately after phosgene exposure.

Phosgene Dose	N (exposed)	N (tolerating halothane & saline)
200 ppm·min	10	9
300 ppm·min	10	8
400 ppm·min	10	7
500 ppm·min	10	8
700 ppm·min	10	6

numbers of rats recovering from treatment and used in assessing treatment effects is given in Table 2. The time of sacrifice varied with the type of treatment effect under examination: (1) mortality / 24 hours after exposure ; (2) lung weights / either 6 or 24 hours after exposure; (3) airway lining fluid composition / 4 hours after exposure; (4) lung function tests / 7 hours after exposure; (5) magnetic resonance imaging / 6 hours after exposure.

The final sample for each of the treatment groups used for assessing mortality effects contained animals from at least eight different phosgene exposures. For assessing other effects, the sample contained animals from at least four different phosgene exposures.

Procedures

Surfactant replacement

Exosurf® [manufacturer's Lot #70257, supplied in 50-ml vials containing 472.5 mg dipalmitoylphosphatidylcholine (DPPC), 52.5 mg cetyl alcohol (a spreading agent), 35 mg tyloxapol (a non-ionic dispersing agent and 204 mg sodium chloride] was obtained from the Burroughs Wellcome Company as a dry powder and was reconstituted with sterile water immediately before use. Exosurf, is a synthetic preparation containing no apoproteins. The cetyl alcohol is used as a substitute for the apoprotein component of natural surfactant to promote rapid spreading and adsorption of the surfactant to the air-liquid interface in the lung. The surfactant replacement was instilled directly into the lungs through an 18-gauge cannula, which was inserted perorally into the trachea of halothane-anesthetized animals with the cannula tip positioned approximately 1 cm above the tracheal bifurcation. The surfactant was rapidly administered in a single bolus, and after removing the cannula, the treated animal was gently rotated between supine and prone positions for approximately 15 seconds, then monitored until recovery from the anesthetic was apparent. Animals which did not recover from the anesthetic were not used in assessing treatment effects or in determining the $LCt_{50,10,24}$ for phosgene.

Table 2: Exosurf® treatment groups. Dosages, numbers of animals killed by the halothane and/or instillation of surfactant and saline, and numbers of animals successfully recovering from instillation and used to assess treatment effects following phosgene exposure and specified treatment.

Treatment	Exosurf® Dose (mg DPPC)*	Volume Instilled (ml)	N Tolerating treatment	N Not tolerating treatment
Controls (Sham treated)	0	1	359	18
Low Dose (immediately after exposure)	13.5	1	78	3
Intermediate Dose (immediately after exposure)	40.5	1	188	2
High Dose (immediately after exposure)	135	1	78	4
High Volume (immediately after exposure)	40.5	2	76	4
Low Volume (immediately after exposure)	40.5	0.5	80	0
Pretreatment (surfactant given before exposure to phosgene)	40.5	1	42	5
Delayed Treatment (given 6 hours after exposure)	40.5	1	20	20

*DPPC: Dipalmitoylphosphatidylcholine

Magnetic resonance imaging

Magnetic resonance imaging (MRI) was used to examine the distribution of instilled surfactant or to characterize the temporal coarse of pulmonary edema in eighteen rats. Immediately after exposure, animals were transported to the MRI laboratory, where anesthesia was induced with brevital (85 mg/kg i.p.) and 1.5% halothane. Animals were then given an injection of atropine sulfate (3 mg/kg i.p.), intubated perorally with an 18-gauge cannula cut to 3 cm and ventilated on halothane (0.5 to 1.5%) at a FiO_2 of 0.21. Imaging was performed with a 30-cm, horizontal-bore 2 Tesla CSI system (General Electric) after placing animals in a small-diameter birdcage coil. Cardiac-gated, multi-slice or multi-echo pulse sequences as previously described [26] were used to image transaxial sections of the animal's thorax with resolutions of 0.1 mm in-plane and 1 to 2 mm out-of-plane.

Once placed in the magnet, a low-resolution, multi-slice image was made to identify the desired axial slice for subsequent high-resolution studies. Once selection was made, all later imaging was done at the same axial level. Animals were repetitively imaged at 20- to 30-minute intervals until death. In analyzing the image set, anatomical references were identified in each image of the series and used to establish that the axial location remained constant. Two regions of well-delineated lung parenchyma per animal were examined, one in the dependent and one in the non-dependent parts of the lung. Signal intensity was averaged over all the pixels in the region using an image analysis program. Temporal changes in signal intensity within these same two regions were used for assessing the onset of pulmonary edema.

Lung function tests

Sixteen animals were exposed to the $LCt_{50,10,24}$ dosage of phosgene. Eight of these were treated with Exosurf® (40.5 mg DPPC/ml, 1-ml intratracheal), and the remaining eight were given saline (1-ml intratracheal bolus). Another set of 16 rats were treated as above with either Exosurf® or saline but were not exposed to phosgene.

Immediately after phosgene exposure, animals from each of the above groups were transported to the lung function testing laboratory and subjected to a series of pulmonary function

tests to ascertain the deterioration/amelioration of lung function over the first several hours after exposure.

The pulmonary function tests were of two types. Immediately after exposure, animals were individually contained within Fenn-type whole-body plethysmographs as previously described [27] in order to assess frequency of breathing (FOB), peak inspiratory (PIF) and expiratory (PEF) flows, and average oxygen consumption. Measurements were made at 30-minute intervals for a period of up to five hours. A second series of pulmonary function tests were performed five hours after exposure to phosgene. Immediately prior to testing, the rats were removed from the Fenn-type plethysmograph, anesthetized with sodium pentobarbital (50 mg/kg i.p.) and intubated with a Luer stub adapter. After paralyzing with succinylcholine (12 mg/kg i.p.), rats were ventilated with a Harvard rodent respirator at 80 breaths/min and a tidal volume equal to their tidal volume during spontaneous breathing. The rats were transferred to a whole-body pressure plethysmograph for in vivo lung function measurements as previously described [28]. Briefly, vital capacity (VC) was measured between airway pressures of -20 and +30 cm water, total lung capacity (TLC) was obtained by gas dilution methods, and residual volume (RV) was computed as the difference between TLC and VC. Single-breath diffusing capacity of carbon monoxide (DLCO) was also measured. A quasistatic pressure-volume curve was used to measure respiratory system compliance (CRS). The curve was obtained by slowly inflating the lungs (3 ml/sec) to TLC and then slowly deflating them to RV.

Lung weights

Animals were sacrificed by decapitation. The lungs were removed from the chest cavity, carefully trimmed, and then blotted on filter paper. Lung wet weights were immediately measured using a Mettler analytical balance. The lungs were subsequently dried for 48 hours to a constant weight in a 100°F oven and reweighed for determining dry weight.

Histology

Hematoxylin- and eosin-stained sections were prepared from samples of fresh lung tissue that had been fixed by immersion in formalin. Sections were obtained from sixty-eight phosgene exposed and/or Exosurf, treated rats.

Bronchoalveolar lavage

After anesthetizing with halothane, the animals were exsanguinated by severing the abdominal aorta and the lungs were collapsed by puncturing the diaphragm. The trachea was exposed and intubated with a 13-gauge cannula secured in place by a ligature. The lungs were then lavaged by injecting a 10 ml volume of saline (approximately 35 ml per kg body weight, or 90% of the TLC). The same wash solution was injected and withdrawn three times and then placed on ice. There was no significant difference in the mean volumes recovered from the lungs of phogene exposed lungs between treatment groups, although recovery in exposed rats was lower than air-exposed rats by about 15%. The recovered lavage fluid was centrifuged at 700 g for 10 minutes to isolate the bronchoalveolar airway cells. The supernatant fractions were placed on ice for further processing as described below. The isolated airway cells were resuspended in 5 ml of Geys balanced salt solution (Microbiological Associates, Bethesda Md). An aliquot of the cell suspension was pelleted on a microscope slide using a cytocentrifuge (Shandon Southern Instruments, Sewickley Pa), dried, and stained with Diff-Quik stain (Harleco, Scientific Products) for enumeration of airway macrophages and PMN leukocytes as previously described [28].

Pulmonary surfactant studies

The cell-free bronchoalveolar lavage fluid (BALF) was centrifuged at 15,000 g x 240 minutes to pellet the pulmonary surfactant (PS). After decanting the supernatant (which was retained for measuring protein and lactate dehydrogenase activity as described below) the PS was re-suspended in 0.9% saline solution and then maintained on ice until analysis. Samples of PS from 8 to 10 exposure cohorts in the same treatment group were pooled in order to have sufficient material for analyzing surface activity. The pooled samples were initially suspended in 300 µl of saline. Immediately before loading the sample into the surfactometer, a 20-µl aliquot of the pooled

surfactant was diluted with 80 µl of either saline or cell-free BALF. A 50-µl sample of this suspension was transferred to the sample chamber of the surfactometer (Pulsating Bubble Surfactometer, Electronetics, Inc.) and left undisturbed for 2 minutes to allow the sample temperature to stabilize at 37°. At 2 minutes, a 0.275-µl bubble was formed within the sample chamber with the air space in communication with the atmosphere through a capillary tube. Thirty seconds later, the bubble was oscillated between 0.275- and 0.7-µL volumes at a frequency of 20 oscillations/minute. Surface tension and pressure were continuously monitored over the next 5 minutes. By 5 minutes, the surface properties of the air-liquid interface of the bubble were stable, and the surface tension signal was digitized over an interval of five oscillations. These data were used to determine average surface tension at minimum and maximum bubble volume and to examine surface tension vs. volume expansion/compression hysteresis characteristics as previously described [29].

A lethal dose of pentobarbital was delivered intraperitoneally. The trachea was intubated with a polyethylene tube (PE190), via this tube a very fine catheter (PE10) was inserted until it pierced the pleura of the lung as previously described by Enhorning et. al. [30]. It was extracted until only 2 mm remained in the lung parenchyma. A pressure transducer measured the resistance that met a steady flow of air through the series of tubes: the PE10-tube, the conducting airway of the lung, and the tracheal tube. The airway resistance was studied for five minutes following the initial drop in resistance or pressure due to the flow of air extruding the columns of liquid blocking the lumen of the airways.

Protein determinations

BALF protein concentrations were determined by the method of Lowry [31] using bovine serum albumin standards.

Determination of lactate dehydrogenase (LDH) activity

Cell-free BALF was mixed with an equal volume of 200 mM potassium phosphate buffer, pH 7.4, containing 10 mM sodium pyruvate at 24°C. LDH activity was determined

spectrophotometrically at 340 nm from the initial rate of oxidation of an added amount (180 nmol) of NADH as previously described [32].

IL-6 Bioassay

BALF IL-6 levels were measured using the bioassay procedure of Van Snick et al [33]. 7TD1 cells were plated at 2 x 10^4 cells/ml in Iscoves + 10% FCS in the presence of known concentrations of human IL-6 (R&D Systems) or rat lavage fluids. Cells were incubated for 4 days at 37° C in 5% CO_2 atmosphere, and the effect of IL-6 on cell proliferation was determined using the hexosaminidase assay of Landegren [34].

Fibronectin ELISA

ELISA plates were coated with 5 ug/ml rat fibronectin (Calbiochem) overnight 4°C. Unknowns and standard concentrations of rat fibronectin were incubated with 1:10,000 dilution of rabbitt anti-rat fibronectin (Calbiochem) overnight at 4°C in polypropylene plates. ELISA plates were washed and antigen antibody mix was added to plates and incubated 10 minutes at room temperature. Plates were washed, and a 1:4000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG (Organon Teknika) was added. The plates were then incubated for 2 hours at room temperature. Plates were washed, and Sigma 104-105 substrate was added at 1 mg/ml. Plates were read at an O.D. of 405.

Aerosol Studies

Animals (Fisher-344 rats) were exposed ingroups of 10 to Exosurf ™ using a cylindrical chamber with a volume of seven liters utilizing a flow-through, nose-only design, following the phosgene exposure. The animals were restrained using the same nose-only restraints used in the phosgene exposure. Ten animals remained in the restraints, on the counter, breathing room air as controls.

There were four exposure regimens used for the Exosurf™ exposures. 1) 6X Exosurf (81mg active ingredient per ml solution), 2) 3X Exosurf (40.5 mg per ml), 3) 3X Exosurf with 1% Poloxamer and 4) 6X Exosurf with 1% Poloxamer. Since no chamber concentration was requested, the highest concentration obtainable based upon the physical characteristics of the Exosurf™ was chosen. All exposure regimens utilized the same methods for aerosol generation and monitoring. The Exosurf was aerosolized using an FDA approved medical nebulizer (TriNEB-400, VORTRAN Medical Technology, Inc., Sacramento, CA) with a liquid reservoir capacity of 240 ml. The nebulizer is a three jet unit, but only one was used for the exposures, was operated with filtered, dried compressed air at 30 psig. The airflow measured with a dry gas meter was 14.5-15.0 liters per minute. After leaving the nebulizer, the aerosolized Exosurf was diluted with 10-15 liters of air from the same source as the nebulizer, to dry the aerosol before entering the exposure chamber. The exhaust from the chamber was HEPA filtered before being exhausted to the atmosphere. Aerosol mass concentration was determined gravimetrically from an extra animal port on the chamber using an open faced holder (Aerosol Open Type Filter Holder, Millipore Filter Corporation, Bedford, MA) connected to the chamber using a custom designed and built adapter. The filtering media was a membrane filter (0.30m mixed cellulose ester, PHWP or 0.40m polycarbonate, HTBP, Millipore Filter Corporation, Bedford, MA). The gravimetric sampling rate was between 0.098 to 2.02 liters per minute, to give a sample collection volume of 2.4 to 30.8 liters. All filters were dried before and after sampling to ensure that the weight change was due to the Exosurf collected. The concentration was calculated by dividing the change in weight (mg) by the volume of air sampled (L) and converted into cubic meters (m^3). Mean chamber concentration of Exosurf was 399 ± 233 mg per m^3. Aerosol particle size and distribution were determined using aerodynamic mass, utilizing two different types of impactors. The first was a real-time cascade impactor (PC-2 QCM Cascade Impactor, California Measurements Inc., Sierra Madre, CA). The second was a Mercer Cascade Impactor (In-Tox Products, Albuquerque, NM) of various size ranges (0.3-3.0 m, 0.25-5.0 m and 0.78-12.65 m). The QCM is a cascade impactor that has a quartz crystal microbalance in each stage. The microbalance determines the frequency

shift between a pair of matched quartz crystals as a function of mass. Air from the exposure chamber is drawn through the impactor and accelerated. At each stage, particles that are of the appropriate aerodynamic mass make a 90 degree turn to stay in the accelerated airstream; particles of the inappropriate aerodynamic mass are impacted on a quartz crystal increasing the mass. Since this device can sense a small change in mass instantly, it can be used to determine particle distribution in real-time. The Mercer impactor is a common method of determining particle size and distribution. Air from the exposure chamber is drawn through the impactor and accelerated. At each stage, particles that are of the appropriate aerodynamic mass make a 90 degree turn to stay in the accelerated airstream; particles of the inappropriate aerodynamic mass are impacted on the preweighed substrate. The substrates are then dried, reweighed, and the weight change calculated. The weight change for both impactors can be converted into mass per volume since the volume of air sampled is known. Both the QCM and Mercer impactor data were analyzed using customized software that calculated the particle size (mass median aerodynamic diameter, MMAD) and distribution (geometric standard deviation, GSD) using "cumulative percent less than basis".

In the first 6X regimen there was some difficulty in controlling the particle size and distribution. This was due to three factors; 1) The 6X Exosurf was viscous and tended to clog the jet of the nebulizer during the exposure, 2) The 6X Exosurf active ingredient concentraton caused a larger particle size and tended to form agglomerates that skewed the distribution data towards larger particle sizes and gave large geometric standard deviations (GSD's), 3) The use of the QCM was inappropriate for these exposures due to the high chamber concentrations. To try to control and more accurately determine the particle size and distribution the following was done; 1) When gravimetric data showed that the operational jet was becoming clogged, the compressed air was connected to a new jet in the nebulizer. 2) Use of the QCM was discontinued in favor of a Mercer impactor. Both of these actions improved the generation and particle size analysis, evidenced by the second 6X exposure regimen which shows a more consistent particle size and a smaller GSD. There was no way control the agglomeration problem with the 6X Exosurf except to dilute the concentration of active ingredients.

The 3X Exosurf regimen was better from generation and monitoring standpoint. The 3X was much less viscous, the active ingredient was more dilute, therefore, did not agglomerate resulting, as expected, in a smaller particle size. Further it meant that a single jet could be used for an entire exposure. The 3x regimen was used for all animals in which aerosol treatment effects were assessed. Mean particle size for these animals was 2.9 ± 0.8 μM.

Phospholipid determinations

Pulmonary surfactant phospholipid was measured by the method of Stewart [35] against a dipalmitoylphosphatidylcholine standard curve after suspending the PS pellet in 3 ml of chloroform and combining with 1 ml of Stewart's reagent (27 g ferric chloride hexahydrate, 30 g ammonium thiocyanate in 1 liter H_2O).

Data Analysis

The graphical method of Litchfield and Wilcoxon (36) was used to estimate the $LCt_{50,10,24}$ dosage for phosgene (3 degrees of freedom, N' = 23 animals).

Effects of surfactant replacement on 24 hour mortality statistics were analyzed using two-way frequency tabulations of 24 hour mortality and survival observations for sham treated, phosgene exposed rats (N = 189) and surfactant treated, phosgene exposed rats ($N \geq 36$). The Pearson chi-square statistic was used to test for differences between expected and observed mortality/survival frequencies in the surfactant treatment groups (2 degrees of freedom). Statistical significance was assumed where $p<0.05$.

The effects of phosgene and surfactant replacement on lung function parameters (FOB, PIF, PEF, oxygen consumption, PIF/PEF ratio, TLC, VC, RV, DLCO CRS) were analyzed by one-way analysis of variance and unpaired t-test.. Lung weights (wet, dry and wet/dry ratio), BALF composition (protein concentration and LDH activity) and PS surface activity for treatment groups were compared using the Student t-test. Significance was assumed where $p < 0.05$.

Group means are expressed in the form mean ± standard error of mean (SEM).

Results

Section 1: Effects of SRT on normal rat lung tissue

Exosurf® was given to healthy, anesthetized rats to determine the dose range that could be safely used in treating phosgene-exposed animals. Two hundred rats were examined for the effects of Exosurf on lung weights and BALF composition. We administered the drug directly into the lungs as a single bolus through an intratracheal cannula in amounts ranging from 13.5 to 135 mg DPPC (48 to 480 mg DPPC/kg body weight) and followed the animals for a period of up to 2 weeks to identify and characterize any adverse treatment effects. Specifically, we examined the effects of Exosurf® treatment on airway lining fluid cellularity, protein content and LDH activity, as well as its effect on lung weight. The results of these studies are summarized in Tables 3 to 7.

Most of the treated animals examined were found to have inflamed airways as indicated by migration of PMN leukocytes into the epithelial lining fluid (Table 3). This inflammatory response was mainly evident 24 hours after treatment; abnormal numbers of airway PMN leukocytes were seen in only a few animals 4 hours after instillation. Interestingly, animals treated with the highest doses of Exosurf® were the least likely to present with elevated airway PMN leukocytes, although histological findings suggested that PMN leukocytes had infiltrated into the lung tissue at all doses. Fourteen days after the drug was administered, the inflammatory response had completely resolved: BALF PMN leukocyte counts had returned to normal levels (< 1% of the recovered airway cells). The same basic pattern was observed in sham-treated rats, i.e., animals that had been instilled with saline instead of the Exosurf® suspension.

Exosurf® instillation had little if any discernible effect on microvascular protein permeability (Table 4). There was no increase in BALF protein content at either 4 hours or 24 hours after treatment at any dose of Exosurf® doses tested (up to 135 mg DPPC). There was no detectable increase in the amount of lactate dehydrogenase enzyme activity in the airway lining fluid at 4 hour after treatment (Table 5). This finding held across the full range of tested doses. However, by 24 hours after treatment, there was an approximately twofold increase in LDH

activity found in the BALF of Exosurf® treated rats. This effect was not dose-dependent and was observed in sham-treated rats. Presumably, the release of this cytosolic marker enzyme into the epithelial lining fluid within the first 24 hours after treatment was triggered by the instillation process and not the drug.

We also found an Exosurf® effect on lung weights 24 hours after treatment with the 40.5 mg Exosurf® dosage, which caused a significant increase in lung wet weight (Table 6) and dry weight as compared to untreated animals (Table 7) The magnitude of the increase in dry weight was in the range of the dry weight of the Exosurf®. The effect of the drug on lung weights was not observed after treating rats with either 13.5 mg or 135 mg Exosurf®.

Tissue sections prepared from Exosurf®-treated lungs 2 weeks after instillation revealed no abnormalities: alveolar septa were uniformly thin and the alveoli were completely empty. There was no evidence of inflammation, hyperplasia or pulmonary edema. These findings held across the entire dose range.

These studies indicate that doses of Exosurf® containing up to 50 mg of phospholipid instilled directly into the lungs are readily tolerated by 300-g rats, eliciting at most a transient inflammatory response which is completely reversible. Higher doses of Exosurf® may be edematogenic, although again the effect is completely reversible in healthy rats.

Distribution of instilled Exosurf

We used proton MRI to analyze the distribution of the instilled surfactant phospholipid within the lung. These studies were once again performed on healthy, anesthetized rats. Prior to instilling the surfactant replacement, the anesthetized rats were placed in the 2 Tesla magnetic field and a series of axial images of the chest were collected. The animal was then removed from the magnetic field, treated with a volume of Exosurf® through an intratracheal cannula and immediately repositioned in the magnet for additional imaging studies.

Photograph 1 is typical of the pretreatment images obtained using the 2D-Fourier transform spin-echo pulse sequences. It clearly shows the outline of the heart and lung parenchyma within the thoracic cavity, and the location of the major airways and vessels. The limited signal arising

Table 3. Effects of surfactant replacement therapy on normal (unexposed to phosgene) rat lung: Airway inflammation. Fractions of treated rats with elevated BALF neutrophil counts 4 and 24 hour after intratracheal instillation of Exosurf®. Animals were lightly anesthetized with halothane before instillation. All surfactant doses given in a volume of 1 ml. Untreated animals were anesthetized but were not intubated. Note: Rats were not exposed to phosgene before or after treatment.

Exosurf® Dose	4 hours after treatment	24 hours after treatment
Untreated	0 of 6	Not Determined
13.5 mg DPPC	1 of 5	3 of 4
40.5 mg DPPC	1 of 5	4 of 5
135 mg DPPC	0 of 3	1 of 3

Table 4. Effects of surfactant replacement therapy on normal (unexposed to phosgene) rat lung: BALF protein concentration. See Table 3 legend for conditions.

Exosurf® Dose	4 hours after treatment*	24 hours after treatment*
Untreated	0.248 ± .048	Not Determined
13.5 mg DPPC	0.205 ± .031	0.226 ± .031
40.5 mg DPPC	0.285 ± .048	0.241 ± .028
135 mg DPPC	0.205 ± .027	0.237 ± .032

*mg protein/ml BALF; Mean ± SEM, N = 4.

Table 5. Effects of surfactant replacement therapy on normal (unexposed to phosgene) rat lung: BALF Lactate Dehydrogenase Activity. See Table 3 legend for conditions.

Exosurf® Dose	4 hours after treatment*	24 hours after treatment*
Untreated	42.6 ± 1.3	Not Determined
13.5 mg DPPC	34.0 ± 1.7	89 ± 14‡
40.5 mg DPPC	37.8 ± 3.3	135 ± 5Δ
135 mg DPPC	28.7 ± 2.3	78.5 ± 22.6‡

*Units: nmol NADH oxidized/ml BALF; Mean ± SEM, N=4 for Untreated & 13.5 mg groups; N=5 for untreated (4 & 24 hour) and 13.5 mg DPPC (4 & 24 hour), N=4 for 40.5 mg DPPC (4 and 24 hour) and 135 mg DPPC (4 hour). N=3 for 135 mg DPPC (24 hour) group. ‡$p \leq 0.05$; Δ$p \leq 0.01$.

Table 6. Effects of surfactant replacement therapy on normal (unexposed to phosgene) rat lung: Lung wet weights. See Table 3 legend for conditions.

Exosurf® Dose	4 hours after treatment*	24 hours after treatment*
Untreated	1.32 ± .04	Not Determined
13.5 mg DPPC	1.25 ± .02	1.23 ± .02
40.5 mg DPPC	1.36 ± .05	1.77 ± .13‡
135 mg DPPC	1.35 ± .05	1.19 ± .02‡

*Units: grams. Mean ± SEM. N=15 for Untreated; N=5 for 13.5 mg DPPC (4 & 24 hour) and 40.5 mg DPPC (4 hour); N=8 for 40.5 mg DPPC (24 hour); N=4 for 135 mg DPPC (4 hour); N=3 for 135 mg DPPC (24 hour) group. ‡$p \leq 0.05$.

Table 7. Effects of surfactant replacement therapy on normal (unexposed to phosgene) rat lung: Lung dry weights. See Table 3 legend for conditions.

Exosurf® Dose	4 hours after treatment*	24 hours after treatment*
Untreated	0.27 ± .01	Not Determined
13.5 mg DPPC	0.26 ± .01	0.26 ± .01
40.5 mg DPPC	0.28 ± .01	0.33 ± .02‡
135 mg DPPC	0.28 ± .01	0.24 ± .01

*Units: grams. Mean ± SEM. N=15 for Untreated; N=5 for 13.5 mg DPPC (4 & 24 hour) and 40.5 mg DPPC (4 hour); N=8 for 40.5 mg DPPC (24 hour); N=4 for 135 mg DPPC (4 hour); N=3 for 135 mg DPPC (24 hour) group. ‡$p \leq 0.05$.

from normal lung parenchyma is due to its low proton density and to a rapid rate of transverse magnetization relaxation.

After administering Exosurf® (13.5 mg DPPC) in 1 ml isotonic saline, we observed an immediate and dramatic increase in the intensity of signal arising from the lung parenchyma. This was expected, since the Exosurf® deposited within the lungs contains not only a rich source of MRI detectable protons, but also a type of proton which tends to have long transverse relaxation times relative to water proton. The heterogeneous distribution of the drug is easily seen in Photograph 2, taken immediately after instillation of the surfactant. Most of the Exosurf® has accumulated in the dependent regions of the right lung lobes. When we increased the volume of saline used to deliver the Exosurf to the lungs, we observed a much more uniform distribution. Photographs 3 and 4 are posttreatment images of different rats that had been treated with larger volumes of Exosurf® (2 or 3 ml saline, respectively, 13.5 mg DPPC per ml). In both cases, images were taken 2 hours after Exosurf® was administered at approximately the same thoracic level using identical imaging conditions (cardiac-gated, end expiratory images, spin echo Fourier transform sequences, echo times of 8 ms and repetition rates of 850 ms). While we still observed some degree of heterogeneity in the former case, with accumulation again appearing in the dependent regions, this was not apparent in the animal treated with 3 ml.

Section 2: Effects of a lethal dose of phosgene gas on rat lungs

Phosgene $LCt_{50,10,24}$

The $LCt_{50,10,24}$ dosage for phosgene was determined from a set of 50 sham-treated rats (animals lightly anesthetized with halothane and given an intratracheal bolus of saline immediately after exposure) exposed for 10 minutes to one of the five following phosgene concentrations: 20, 30, 40, 50 or 70 ppm. The results of these studies are presented in Table 8. The estimated $LCt_{50,10,24}$ was 405 ppm·minutes. The $LCt_{50,10,24}$ dosage was also determined in a set of 50 untreated exposure cohorts (animals that received no anesthesia or saline following exposure) and found to be 437 ppm-minutes.

Table 8. LCT_{50} determination: Phosgene 10-minute exposures, 24-hour survival. Immediately after exposure, rats were individually anesthetized with halothane and given 1 ml of a 0.9% saline solution by intratracheal instillation.

Phosgene Dose (ppm·min)	N	24-hour Mortality
200	9	0%
300	8	38%
400	7	57%
500	8	50%
700	6	100%

N=number of phosgene exposed rats surviving saline instillation and recovering from halothane.

Effect of the $LCt_{50,10,24}$ dosage of phosgene on lung weights

The temporal course of lung wet weight changes following the 10-minute exposure to the $LCt_{50,10,24}$ phosgene dosage is shown in Figure 2. Normal lung wet weight is 1.27 ± 0.03 g, as determined from a group of fifteen unexposed cohorts. One hour after exposure, lung wet weights were already slightly higher than normal (1.65 ± 0.09 g, N=10). No further gains were evident over the next hour (2-hour postexposure wet weight = 1.68 ± 0.08 g, N=12). At 4 hours, average wet weight had again increased, rising to 1.81 ± 0.10 g (N=12), a gain of 0.13 g. The greatest change in lung wet weight came between 4 and 6 hours, up to 2.33 ± 0.17 g (N=11), a net gain of 0.52 g. By 24 hours after exposure, average wet weight was 2.68 ± 0.05 g (N=69). The net increase in wet weights from 6 to 24 hours was 0.35 g.

The temporal course of lung dry weight changes is shown in Figure 3. Dry weights 1 and 2 hours after exposure to the $LCt_{50,10,24}$ dosage were within the normal range, 0.27 ± 0.02 (N=10) and 0.26 ± 0.01 g (N=12) vs. a normal value of 0.26 ± 0.01 g (N=15). A small gain in dry weight occurred between 2 and 4 hours after exposure, up 0.04 g to 0.30 ± 0.01 g (N=12). A further increase occurred between 4 and 6 hours, up 0.07 g to 0.37 ± 0.02 g (N=11). The greatest change in dry weights occurred over the subsequent 18 hours. By 24 hours after exposure, lung dry weights rose to 0.52 ± 0.01 g (N=67), an increase of 0.15 g.

These studies indicate that most of the edema fluid which would enter the lung in response to phosgene is already in the tissue 6 hours after exposure. At this time, lung water content was already nearly twice its normal value, up to 1.96 ml vs. its normal 1.01 ml, a net gain of 0.95 ml. Over the next 18 hours, lung water would rise to 2.16 ml, an additional 0.20 ml over the 6-hour levels.

Figure 2
Effect of phosgene on lung wet weight.
Animals exposed to 40.5 ppm phosgene for 10 minutes. Immediately after exposure, rats were individually anesthetized with halothane and given 1 ml of a 0.9% saline solution by intratracheal instillation. All weights were significantly elevated relative to normal lung wet weight (1.27±0.03 g) determined in a group of cohorts receiving no phosgene or saline.

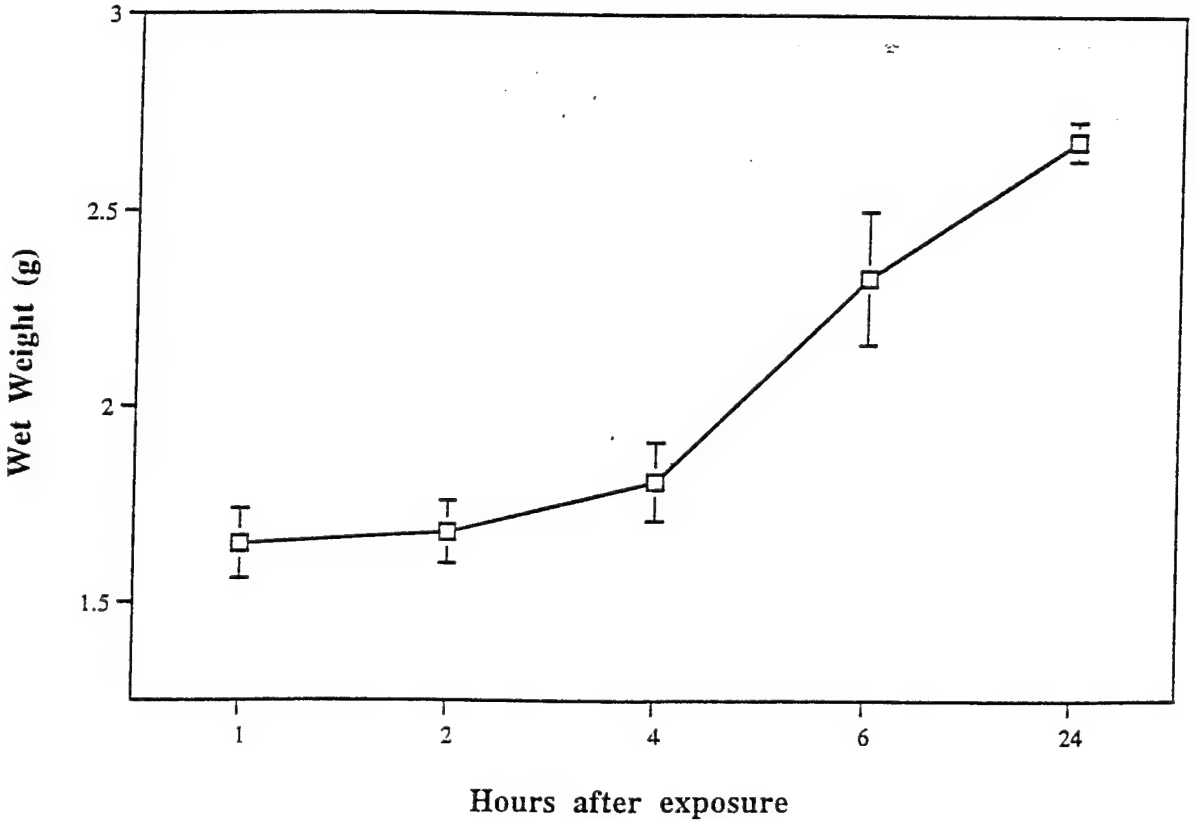
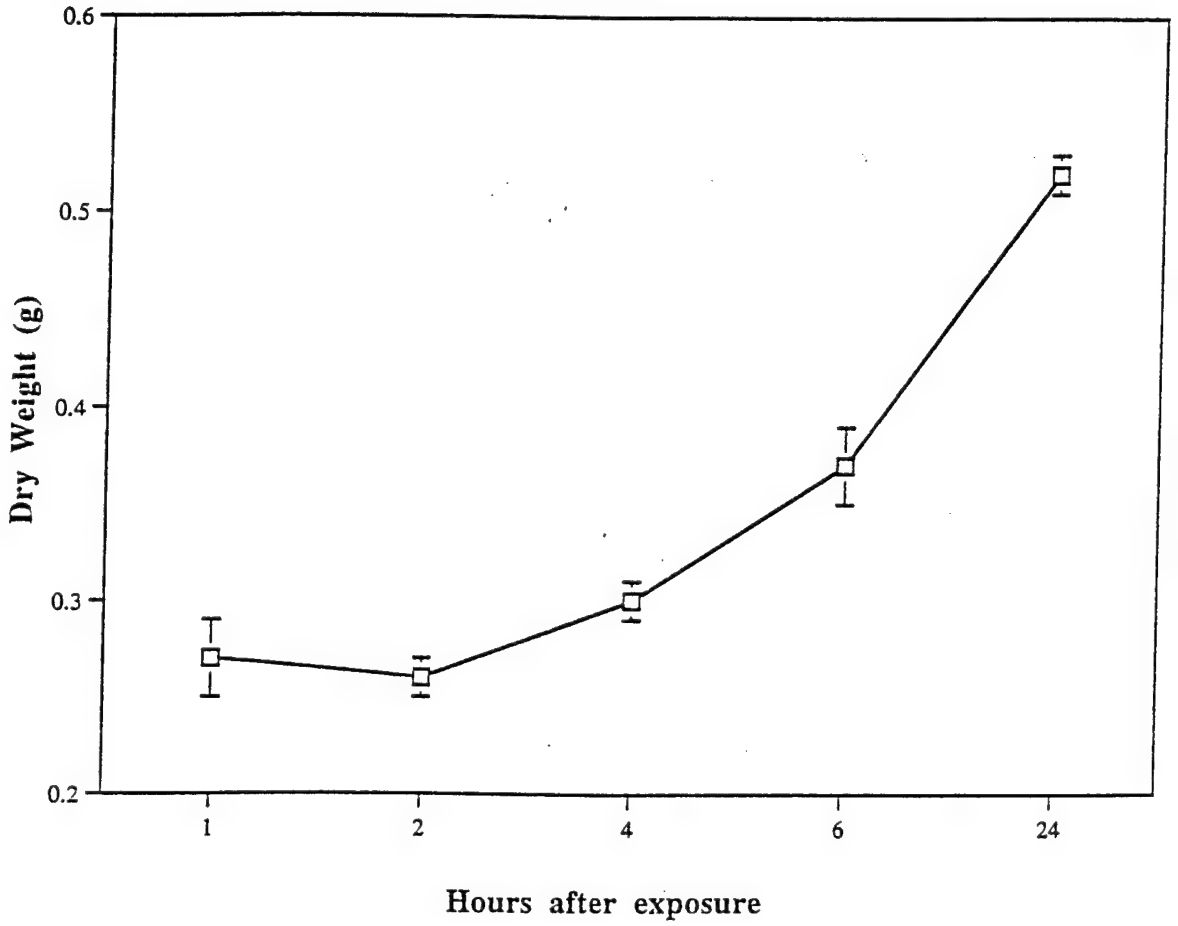


Figure 3
Effect of phosgene on lung dry weight.
Animals exposed to 40.5 ppm phosgene for 10 minutes. Immediately after exposure, rats were individually anesthetized with halothane and given 1 ml of a 0.9% saline solution by intratracheal instillation. Normal lung dry weight was 0.26±0.01 g as determined in a group of cohorts receiving no phosgene or saline.



Effect of the $LCt_{50,10,24}$ dosage of phosgene on epithelial lining fluid composition

One hour after exposure, BALF protein levels were still within the normal range. Over the next hour, protein levels rose significantly from a normal value of 0.22 ± 0.08 to 2.1 ± 0.4 mg/ml. However, the bulk of the protein leaked into the epithelial lining fluid between 2 and 4 hours after exposure. Four hours after exposure, BALF protein concentration rose to 9.5 ± 2.2 mg/ml. There was no significant change in BALF protein from 4 to 6 hours after exposure (Figure 4).

The only other composition change observed 4 hours after exposure (Table 9) involved BALF lactate dehydrogenase activity which was more than twice its normal value (85.9 ± 5.3 vs. 42.6 ± 1.2 nmol NADH oxidized/min). There was no discernible effect of phosgene on the amount of PS phospholipid recovered in the BALF.

Effect of the $LCt_{50,10,24}$ dosage of phosgene on PS surface activity

Pulmonary surfactant isolated from the phosgene-exposed rats 4 hours after exposure and suspended in saline rapidly lowered the surface tension during bubble compression; typically, values for surface tension at bubble volume of 0.275 µl were less than 2 mN/m after 100 compression / expansion cycles. This was not significantly different than the value observed with surfactant isolated from normal rats and tested under identical circumstances. In contrast, we found that the surface properties of the PS isolated from the exposed animals were markedly abnormal when we mixed the surfactant with the protein-rich BALF (Table 10). Under these conditions, surface tension at the 0.275-µl volume averaged in excess of 20 mN/m after 100 compression/expansion cycles. This was not significantly different from the value observed with surfactant isolated from normal rats if mixed with cell free BALF from an exposed rat.

The temporal course of the effect of phosgene on the surface activity of isolated surfactant is presented in Figure 5. In all cases, the surfactant was mixed with BALF, and the bubble used to assess surface tension was subjected to 100 compression / expansion cycles before assessing surface tension at a bubble volume of 0.275 µl. There was no significant difference between the surface tension observed 4 and 6 hours after exposure.

Figure 4

Effect of phosgene on BALF protein.

Animals exposed to 40.5 ppm phosgene for 10 minutes. Immediately after exposure, rats were individually anesthetized with halothane and given 1 ml of a 0.9% saline solution by intratracheal instillation. Normal BALF protein concentration was 0.22±0.01 mg/ml as determined in a group of 15 cohorts receiving no phosgene or saline.

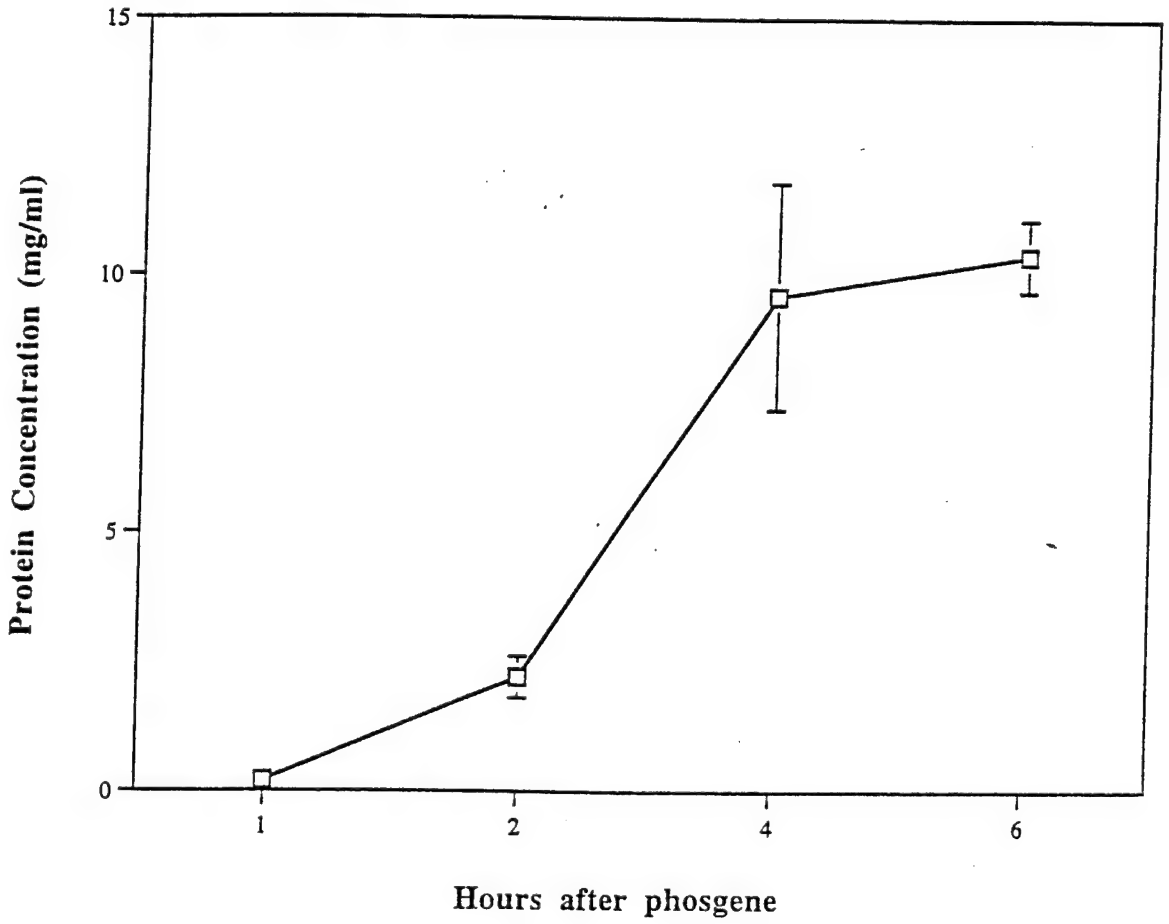


Figure 5

Effect of phosgene on PS surface activity.

Animals exposed to 40.5 ppm phosgene for 10 minutes. Immediately after exposure, rats were individually anesthetized with halothane and given 1 ml of a 0.9% saline solution by intratracheal instillation. PS isolated from BALF at the indicated times and suspended in BALF. Surface tension measured using pulsating bubble technique at minimum bubble radius after 100 pulsations (5 minutes).

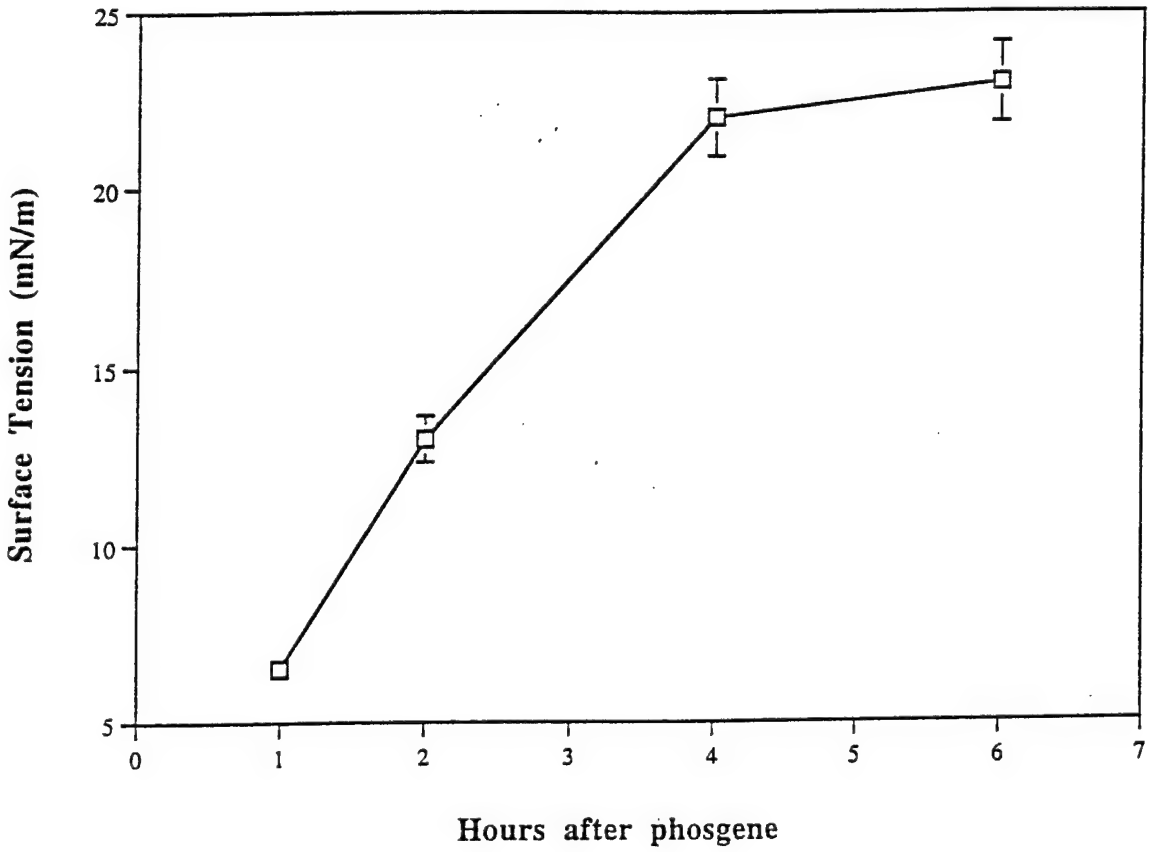


Table 9. Effects of phosgene on BALF composition. Rats were exposed to 40.5 ppm phosgene for 10 minutes. Lung lavages were performed 4 hours after exposure.

	Normal Lung	Exposed Lung
LDH activity nmol NADH oxidized/min/ml	42.6 ± 1.2 (N=6)	85.9 ± 5.3‡ (N=18)
Protein concentration (mg/ml BALF)	0.25 ± .04 (N=6)	8.2 ± 0.9Δ (N=18)
Pulmonary surfactant (mg phospholipid)	0.28 ± .01 (N=19)	0.33 ± .02 (N=7)

Values represent mean ± SEM. Number of observations given in parentheses. Significantly different from normal lung value: ‡$p \leq 0.05$; Δ$p \leq 0.01$.

Table 10. Effects of phosgene on PS surface activity. Rats were exposed to either air or 40.5 ppm phosgene for 10 minutes. Lung lavages were performed 4 hours after exposure. PS was isolated from the BALF by centrifugation and suspended in BALF to a concentration of 2 mg phospholipid/ml. Surface tension was measured using a pulsating bubble surfactometer by the method of Enhorning [29] at minimal bubble radius after 100 bubble pulsations (5 minutes).

Source of PS	Source of BALF used to suspend PS	N	Surface Tension*
Air exposed rats	Air exposed rats	5	10.4 ± 3.6
Phosgene exposed rats	Phosgene exposed rats	6	23.5 ± 3.8‡
Air exposed rats	Phosgene exposed rats	3	26.7 ± 1.7‡

*mN/m. Values represent means ± SEM. Number of observations given in parentheses.
‡Significantly different than normal (PS from air exposed rats suspended in BALF from air exposed rats) $p \leq 0.05$.

Effect of the $LCt_{50,10,24}$ dosage of phosgene on lung function

The temporal courses of the effects of phosgene on lung function are summarized in Figures 6 through 10. Phosgene inhalation affected virtually all measured parameters. The initial battery of tests demonstrated that the effects of phosgene inhalation were apparent within minutes of exposure. Phosgene inhalation was associated with a significant and sustained effect on the frequency of breathing (FOB), peak inspiratory and expiratory flows (PIF & PEF), PIF/PEF ratio, peak expiratory/mean expiratory ratio (PE/ME) and oxygen consumption. The precipitous decline in FOB in the air exposed controls (saline and Exosurf® treated, no phosgene) is presumably due to the removal of the immobilized animal from the treatment chamber and recovery from the associated stress response over time.

Direct measures of lung elasticity performed at 5 hours after exposure confirmed that phosgene had a profound effect on lung function (Tables 11 and 12). Abnormal findings associated with phosgene inhalation included decreased tidal volume, inspiratory and expiratory times, total lung and vital capacity, residual volume, end expiratory volume, CO diffusion capacity and respiratory system compliance.

MRI imaging studies with rats exposed to an $LCt_{50,10,24}$ dosage of phosgene

The noninvasive nature of MRI combined with its sensitivity to pulmonary water content has made it an ideal tool for examining the evolution of pulmonary edema following phosgene exposure. We have been able to image rats as early as 90 minutes after exposure, before the major outpouring of edema water detected in our lung weight determination, which occurs between 2 and 6 hours after exposure. As a result, we have been able to follow both the spatial and temporal progression of the edema. In our studies to date, we have encountered two basic patterns.

In one case, an extensively damaged lung is already apparent in the first image. Most often, the injury is diffuse, with patches of high signal intensity bordered by areas of normal signal strength. Typically, the physiologic status of these animals deteriorates rapidly. Postmortem examination of these animals usually shows a massively edematous lung. In the second and most common case, we see a fairly normal lung in the first image (Photographs 5-7). In subsequent

Figure 6

Effects of phosgene and SRT on frequency of breathing (FOB). Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® or 0.9% saline by intratracheal instillation. Each point represents the mean of 8 observations. Significant differences in group means were found for phosgene exposed vs. air exposed animals (one-way ANOVA) and phosgene exposed, saline treated vs. phosgene exposed, Exosurf® treated animals (paired t-test) during the first 5 hours after exposure. Note : Error bars omitted for clarity.

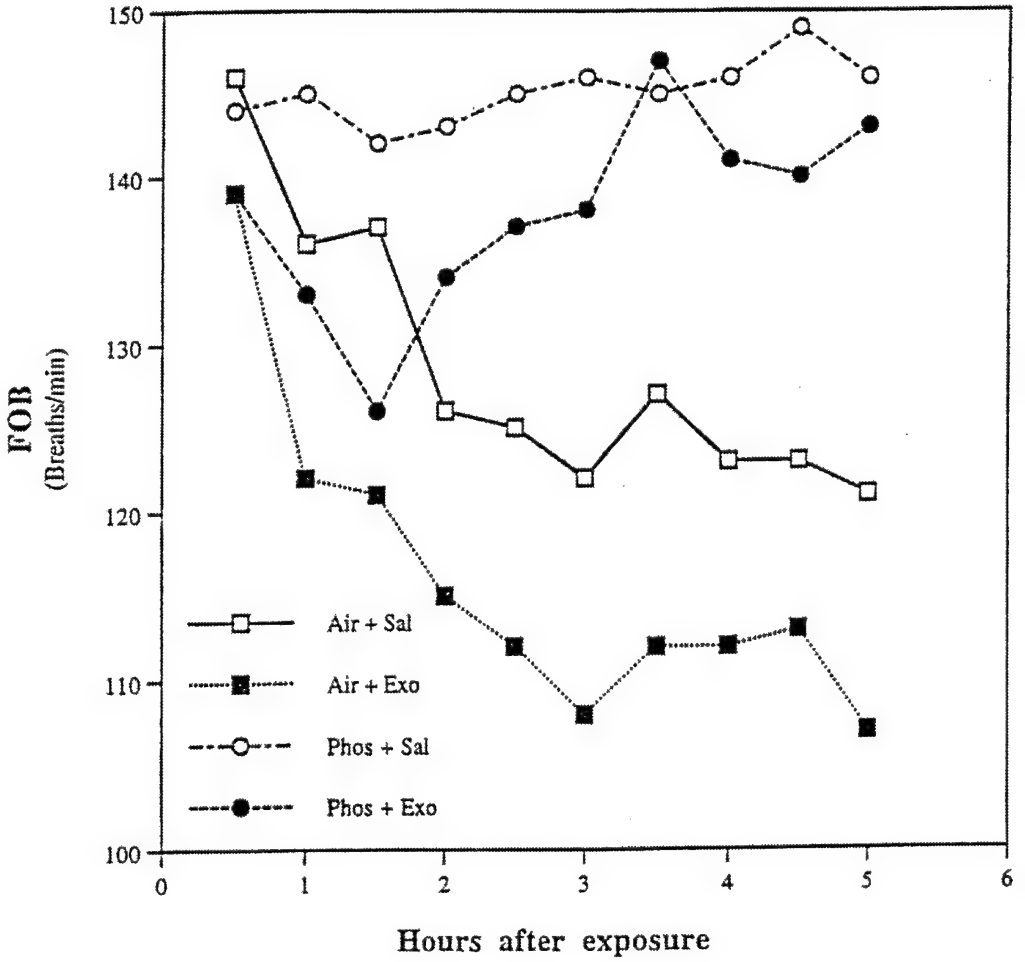


Figure 7
Effects of phosgene and SRT on peak inspiratory flow (PIF).
Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® or 0.9% saline by intratracheal instillation. Each point represents the mean of 8 observations. Significant differences in group means were found for phosgene exposed vs. air exposed animals (one-way ANOVA) and phosgene exposed, saline treated vs. phosgene exposed, Exosurf® treated animals (paired t-test) during the first 5 hours after exposure. Note: Error bars omitted for clarity.

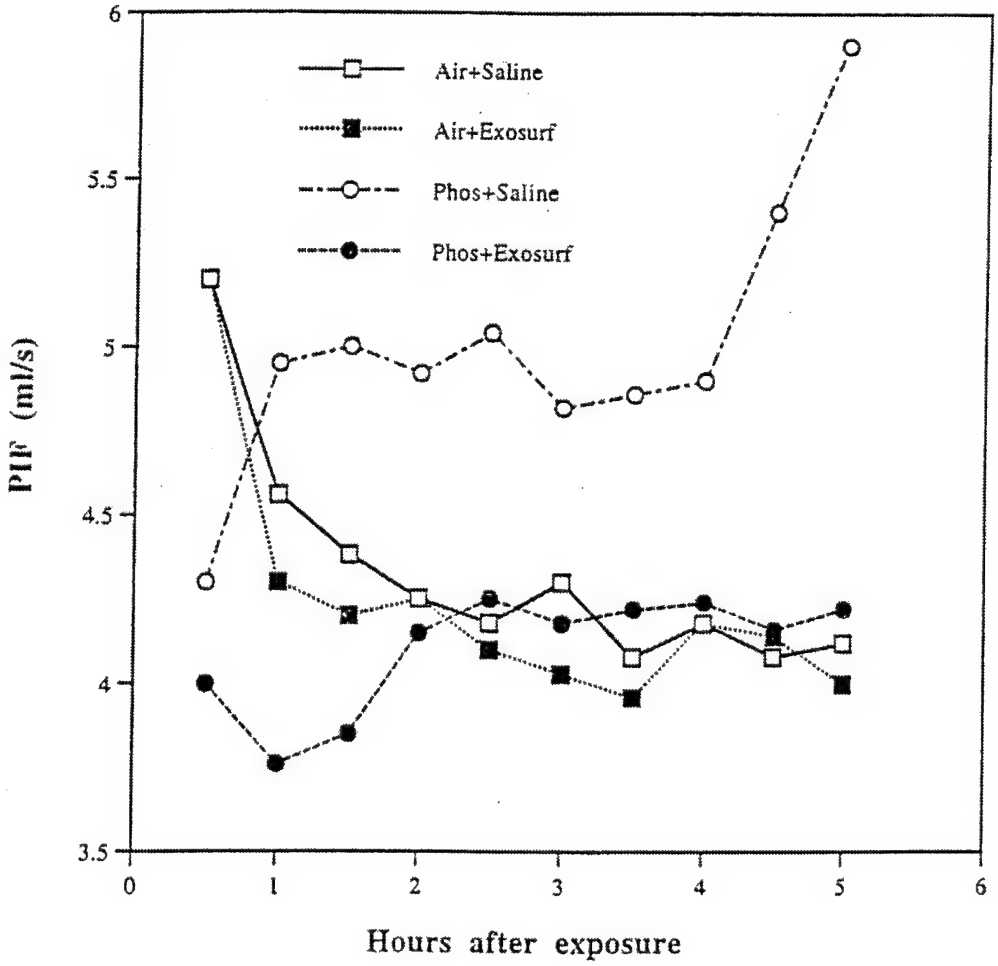


Figure 8
Effects of phosgene and SRT on peak expiratory flow (PEF).
Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® or 0.9% saline by intratracheal instillation. Each point represents the mean of 8 observations. Significant differences in group means were found for phosgene exposed vs. air exposed animals (one-way ANOVA) and phosgene exposed, saline treated vs. phosgene exposed, Exosurf® treated animals (paired t-test) during the first 5 hours after exposure. Note: Error bars omitted for clarity.

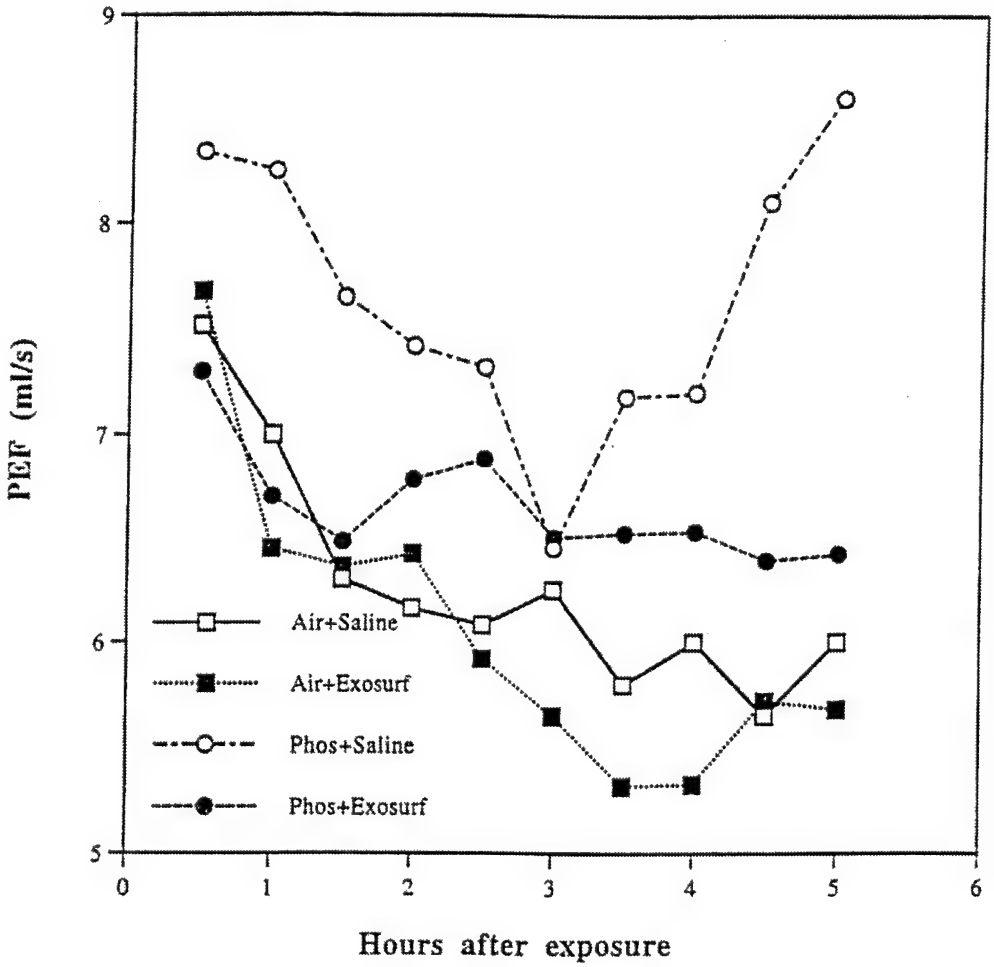


Figure 9
Effects of phosgene and SRT on oxygen consumption.
Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® or 0.9% saline by intratracheal instillation. Each point represents the mean of 8 observations. Significant differences in group means were found for phosgene exposed vs. air exposed animals (one-way ANOVA) during the first 5 hours after exposure. Note: Error bars omitted for clarity.

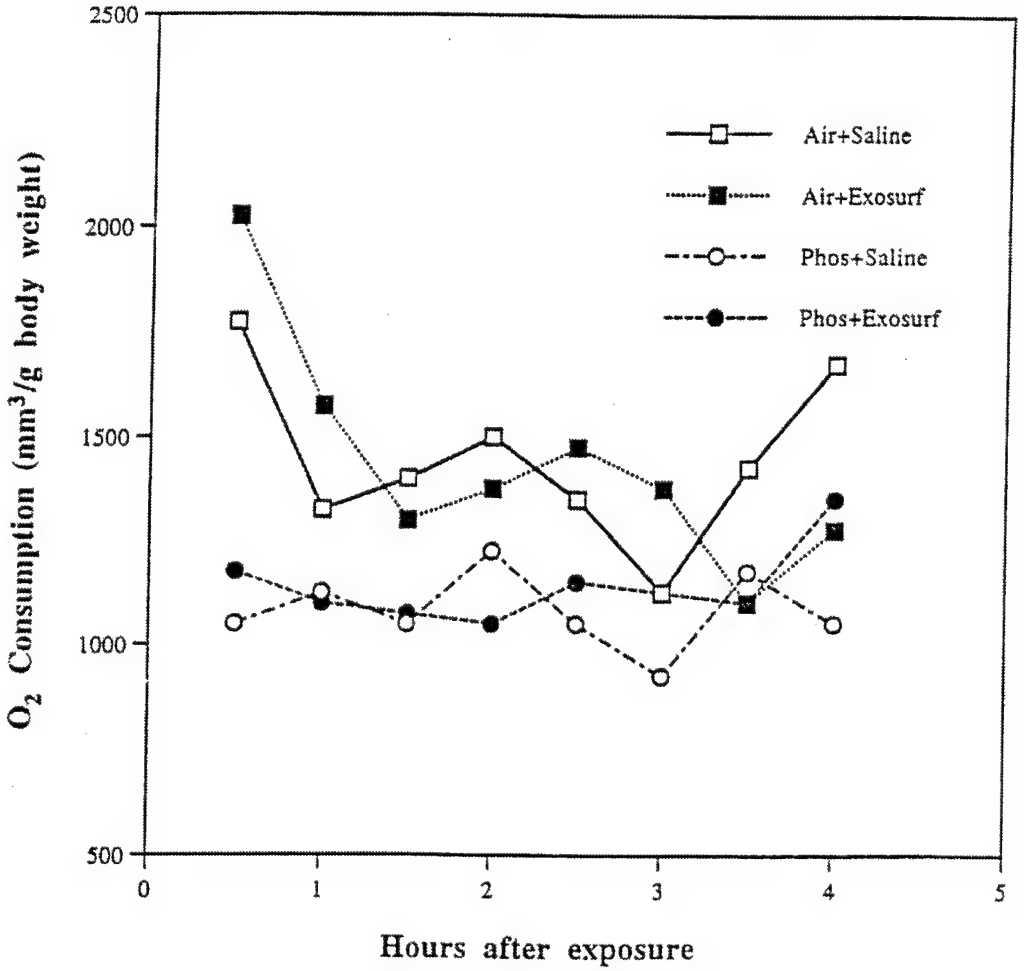


Figure 10
Effects of phosgene and SRT on peak/mean expiratory flow ratio (PEF/MEF)

Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® or 0.9% saline by intratracheal instillation. Each point represents the mean of 8 observations. Significant differences in group means were found for phosgene exposed vs. air exposed animals (one-way ANOVA) during the first 5 hours after exposure. Note : Error bars omitted for clarity.

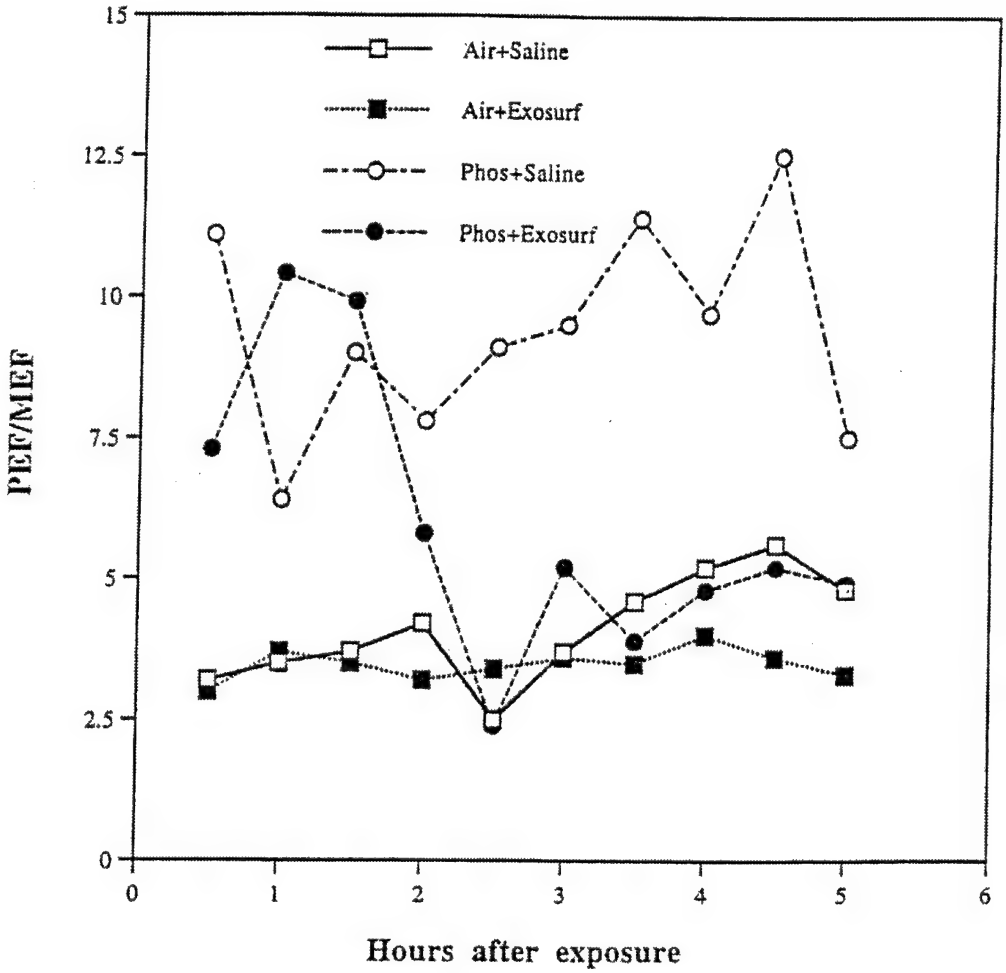


Table 11. Effects of phosgene and surfactant replacement on lung function parameters 5 hours after exposure/treatment. Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® (40.5 mg DPPC), Exosurf® + Poloxamer (40.5 mg DPPC + 1% Poloxamer 188) or 0.9% saline by intratracheal instillation. Lung function measurement were made five hours after exposure/treatment.

Exposure: / **Treatment:**	Air / Saline	Air / Exosurf®	Phosgene / Saline	Phosgene / Exosurf®	Phosgene / Exosurf® Poloxamer
Breaths/min	100 ± 7	98 ± 6	140 ± 8‡	150 ± 4‡	138 ± 9‡
Inspiratory time (seconds)	0.30 ± .01	0.32 ± .02	0.22 ± .02‡	0.22 ± .02‡	0.20 ± .01‡
Expiratory time (seconds)	0.23 ± .01	0.25 ± .01	0.20 ± .02‡	0.17 ± .01‡	0.19 ± .01‡
Tidal volume (ml)	1.61 ± .14	1.88 ± .14	1.36 ± .07‡	1.46 ± .10‡	1.55 ± .08‡
DL_{CO} (ml/min*Torr)	0.175 ± .015	0.215 ± .010	0.070 ± .015‡	0.045 ± .005‡	NA
C_{RS} (ml/cm H_2O)	0.59 ± .07	0.59 ± .06	0.25 ± .06‡	0.16 ± .03‡	0.34 ± .03‡§
N_2 Slope (slope log % N_2/number breaths)	-0.27 ± .03	-0.26 ± .03	-0.26 ± .04	0.25 ± .03	0.14 ± .03
N_2 EEV (ml)	2.77 ± .39	3.00 ± .37	1.52 ± .20‡	2.01 ± .40‡	3.92 ± .42‡

Values represent means ± SEM for 8 observations. ‡ indicates significant difference between phosgene exposed and air exposed cohorts, $p \leq 0.05$. § indicates significant difference between surfactant treated and untreated groups. NA = Not Applicable.

Table 12. Effects of phosgene and surfactant replacement on lung volumes 5 hours after exposure/treatment. Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 1 ml of either Exosurf® (40.5 mg DPPC) Exosurf® + Poloxamer (40.5 mg DPPC + 1% Poloxamer 188) or 0.9% saline by intratracheal instillation. Lung volumes (ml) were assessed five hours after exposure/treatment.

Exposure: **Treatment:**	Air Saline	Air Exosurf®	Phosgene Saline	Phosgene Exosurf®	Phosgene Exosurf® Poloxamer
TLC	11.9 ± 0.8	11.8 ± 0.3	6.8 ± 0.7‡	6.0 ± 0.2‡	NA
RV	1.7 ± 0.2	1.6 ± 0.2	1.5 ± 0.2‡	1.2 ± 0.1‡	NA
VC	10.2 ± 0.7	10.2 ± 0.3	5.3 ± 0.6‡	4.8 ± 0.3‡	4.8 ± 0.4‡
Boyle's Volume	4.1 ± 0.3	3.7 ± 0.2	3.6 ± 0.2	3.7 ± 0.4	3.6 ± 0.2

Values represent means ± SEM for 8 observations. ‡ indicates significant difference between phosgene exposed and air exposed cohorts, $p \leq 0.05$. NA = Not Applicable.

images, however, areas of high signal intensity emerge, giving the appearance in the 2D slice of a focal lesion surrounded by normal tissue. Eventually, if the animal survives long enough, additional areas of increasing signal intensity appear. Three examples have been included in this report.

In the first example, a series of images taken at approximately 30-minute intervals of the same axial slice is presented (Photograph 5). In the first image in this set, several small areas of high intensity are distributed through the right lung. Some of these areas become progressively larger with time, until by the last image the pattern of edema appears relatively diffuse.

In the second example (Photograph 6), three pairs of images from the same animal are presented. The top image was taken approximately 2 hours after exposure and the bottom image some 2 hours later. In slice A, most of the change in signal intensity was confined to two regions within the right lung. In slices B and C, the edematous regions are more diffuse.

In the final example (Photograph 7), another time series taken at 30 minute intervals is displayed which follows the emergence of a relatively focal lesion in the upper right lung.

Effect of $LCt_{50,10,24}$ dosage of phosgene on morphology

H&E stained sections of lung tissue from rats sacrificed 24 hours after exposure to the $LCt_{50,10,24}$ dosage of phosgene consistently showed diffuse edema, with patches of normal tissue interspersed with areas of alveolar septal thickening, alveolar flooding and atelectasis or areas of hemorrhage. Perivascular edema was apparent throughout the tissue. In a few of the animals, edema fluid was also apparent in the bronchi. PMN leukocytes were found in all the sections, primarily in association with the edema fluid.

Section 3: Surfactant replacement therapy as a countermeasure against lethal doses of phosgene.

Effect of SRT on phosgene mortality rates

The effect of SRT on 24-hour mortality following a 10-minute exposure to 40.5 ppm phosgene is summarized in Table 13. One hundred eighty nine sham-treated, phosgene-exposed animals have been followed for 24 hours to determine mortality rates. Eighty-nine of these animals survived to 24 hours; a mortality rate of 53%.

In our initial series of studies examining the effects of SRT on phosgene mortality rates, exposed rats were treated within 30 minutes after phosgene exposure to one of three different doses of Exosurf® (13.5, 40.5 or 135 mg DPPC). All three doses were delivered in the same 1-ml volume of carrier and instilled directly into the lungs. A significant increase in survival was found in the 40.5-mg Exosurf® treatment group: 24 of 38 animals receiving this dose were alive at 24 hours, a mortality rate of 37%. This decrease in mortality was statistically significant (chi-squares test, $p \leq .05$). This was the only dose of the three tested that protected the phosgene-poisoned rats. The mortality rate for animals treated with the lower dose of Exosurf® was 58%, slightly higher than in sham-treated, phosgene-exposed rats. Only 15 of 38 animals treated with the higher dose of Exosurf® (135 mg DPPC) survived. The 61% mortality was the highest we observed in any test group over the first half of this contract, including the sham-treated animals.

In the second series of this project, we addressed the question of whether treatment timing would influence the effectiveness of Exosurf® in reducing mortality rates. In the first part of this series, animals were treated with Exosurf® (40.5 mg DPPC) immediately prior to phosgene exposure. Again, the dose was administered by instillation in a 1-ml volume. Twenty-eight of 40 pretreated animals survived to 24 hours, a mortality rate of 30%. This represented a net difference in mortality percentage of 23% compared to sham-treated animals, a statistically significant finding ($p \leq .01$). In the second part of this series, we treated the animals 6 hours after phosgene exposure

Table 13. Effects of surfactant replacement on 24-hour survival after phosgene exposure. Animals were exposed to 40.5 ppm phosgene for 10 minutes and treated with either saline (sham treated) or Exosurf® by intratracheal instillation as indicated. Treatments were given immediately after exposure unless otherwise indicated. Animals which died during instillation were not considered in assessing the effects of SRT on phosgene mortality.

Treatment	Exosurf® Dose (mg DPPC)*	Volume Instilled (ml)	N Tolerating treatment	Alive @ 24 Hours	Dead @ 24 Hours	24-hour Mortality
Sham treated	0	1	189	89	100	53%
Exosurf (Instilled)						
Low Dose	13.5	1	38	16	22	58%
High Dose	135	1	38	15	23	61%
Low concentration	40.5	2	36	16	20	56%
Standard concentration	40.5	1	38	24	14	37%
High concentration	40.5	0.5	36	25	11	31%
Pretreatment	40.5	1	40	28	12	30%
Delayed Treatment	40.5	1	40	25	15	38%
Multiple Treatments	91	1	40	15	25	63%
Exosurf (Instilled) +						
Vitamin E added	40.5	0.5	40	25	15	38%
1% Poloxamer 188 added	40.5	0.5	39	28	11	28%
Exosurf (Aerosol)						
Sham treated			90	40	50	56%
1 hour treatment			20	14	6	30%
2 hour treatment			20	11	9	45%
4 hour treatment			68	36	32	47%
Other Surfacts/Agents						
Infasurf	20	0.5	50	34	16	32%
Pure DPPC	40.5	0.5	30	17	13	43%
Tyloxapol	0	0.5	30	15	15	50%

Treatment effects analyzed by Pearson's chi-square test vs. sham treated group. Significance levels: $^{\ddagger}p \leq 0.05$; $^{\Delta}p \leq 0.01$

with the same dose of Exosurf® (40.5 mg DPPC). Twenty-six of forty-two of these animals survived to 24 hours, a mortality rate of 38%. This was almost identical to the rate observed in animals treated immediately after exposure and was significantly different from the mortality rate found in sham-treated animals.

In the next series of experiments, Exosurf® was administered to phosgene-exposed rats in different concentrations, 20.25 or 81 mg DPPC/ml. In both cases, the dose of Exosurf® was kept constant (40.5 mg DPPC) and administered to the rats immediately after exposure. Animals treated with the higher concentration (and smaller volume) again benefited from the treatment. Twenty-five of 36 of these animals survived to 24 hours, a mortality rate of 31%. This decrease in mortality relative to sham-treated animals was statistically significant ($p \leq .01$). We did not observe a significant effect of treatment in animals given the lower concentration (and greater volume) of Exosurf®. Only 16 of 36 animals so treated survived to 24 hours, a 56% mortality rate that was slightly worse than the rate observed in sham-treated rats.

The lipophilic antioxidant vitamin E (alpha-tocopherol succinate) was combined with Exosurf prior to instillation into phosgene-exposed rats to supplement endogenous tissue antioxidant levels. The supplement had little if any salutary effect on survival. Once again, we found that animals treated with the surfactant replacement immediately after exposure were more likely to survive to 24 hours. Twenty-five of the forty rats treated with Exosurf + vitamin E (40.5 mg DPPC + 12 IU vitamin E, administered by intra-tracheal instillation as a 0.5 ml bolus) immediately after exposure survived to 24 hours (37% mortality at 24 hours). This survival rate was slightly lower than that observed in animals treated with Exosurf alone under similar conditions.

We also examined the effects of supplementing Exosurf with Poloxamer 188, a synthetic surfactant that antagonizes the inhibitory effects of serum proteins on the surface activity of isolated rat pulmonary surfactant. Forty phosgene exposed rats were treated with a mixture of Exosurf and Poloxamer (1%Poloxamer (w/v), 40.5 mg Exosurf, administered by instillation in a 0.5 ml volume of saline immediately after exposure. Thirty-nine of these animals tolerated the treatment and were

followed for 24 hours to assess the effects of treatment on mortality and pulmonary edema. As indicated in Table 13, twenty-eight of the rats survived (28% mortality at 24 hours). The survival rate in this group was the highest observed in any of the treatment groups, although it was not statistically different from the rate observed with Exosurf alone.

We tested a second commercially available surfactant replacement, Infasurf, in rats exposed to an $LCt_{50,10,24}$ dosage of phosgene. Infasurf is a natural surfactant preparation and contains surfactant specific proteins SP-B and SP-C. Recent studies have demonstrated that surfactant replacements containing these proteins are more effective than Exosurf in maintaining lung function and in alleviating some forms of lung injury.[37-38] However, we did not observe any difference between Infasurf and Exosurf with respect to reducing the lethality of the $LCt_{50,10,24}$ phosgene dosage. Thirty-four of the fifty animals treated with Infasurf (20 mg DPPC administered immediately after exposure by instillation in a volume of 0.5ml survived). The 32% mortality rate observed in this treatment group was nearly identical to that observed in animals treated with Exosurf (31% mortality), and slightly above that observed with Exosurf supplemented with 1% Poloxamer 188 (28% mortality). All treatments effectively counteracted the lethal effects of phosgene exposure.

In our final series, we instilled either 40.5 mg of DPPC or 3 mg of tyloxapol in a volume of 0.5 ml to phosgene exposed animals. The amounts of these two agents are identical to that found in the standard dosage of Exosurf used in these studies. Seventeen of 30 animals treated with pure DPPC and 15 of 30 animals treated with tyloxapol survived. Neither treatment was significantly different from sham treatment.

We found that the surfactant replacement could also be administered as an aerosol to counteract the lethal effects of the $LCt_{50,10,24}$ phosgene dosage. Rats were treated in a Cannon type (nose only) exposure chamber containing aerosolized Exosurf® + Poloxamer 188 (399 ± 233 mg) surfactants per m^3, particle size 2.9 ± 0.8 μM). In order to measure the relative amounts of drug delivered to the lungs by aerosol and by direct instillation, the surfactant replacement was spiked with trace amounts of delta tocopherol, a lipophilic substance that is not normally present or

metabolized in the lung. Within the first ten minutes after instilling the surfactant replacement into the lungs of phosgene exposed rats,we were able to recover 31±8% of the instilled surfactant from the lungs. Most (30±8%) was recovered from the airways by lavage. Only 1.0±0.3% of the instilled surfactant remained in the lung after lavage. By four hours after administration, only 16±3% of the instilled marker was found in the lungs. At this time, only 5±2% could be recovered by airway lavage. The remainder of the surfactant marker (11±2%) had absorbed into the lung parenchyma. The lungs of animals treated by breathing aerosolized surfactant for 4 hours contained 22% of the amount found in the instilled animals. In the aerosol treated animals, about 80% of the surfactant marker was retained in the lungs after lavaging.

Forty of the ninety sham treated rats in our aerosol series survived to 24 hours, a mortality rate of 56%. This was slightly, but not significantly, different from the 53% mortality rate observed in sham treated rats in the instillation series (i.e., animals receiving an intratracheal bolus of saline). Animals treated with aerosolized Exosurf + Poloxamer for one hour immediately after exposure were significantly more likely to survive to 24 hours. Fourteen of twenty animals so treated survived, a mortality rate of 30%. This was similar to that observed in animals treated with Exosurf+Poloxamer by instillation (28% mortality). Longer treatment times appeared to have an adverse effect on the exposed animals. Only 11 of 20 rats treated with the aerosol for 2 hours after exposure survived to 24 hours, a mortality rate of 45%. Extending the treatment time to four hours further increased 24 hour mortality rates. Thirty-six of sixty eight animals so treated survived to 24 hours, a mortality rate of 47%, still slightly, but not significantly different from sham treated animals.

Effects of SRT on lung weights 6 hours after exposure to phosgene

The effects of SRT on lung weights 6 hours after exposure to the $LCt_{50,10,24}$ dosage of phosgene are summarized in Table 14. At the 6-hour point, lung wet weights for various SRT treatment groups ranged from 2.07 ± 0.10 g in animals treated with Exosurf® + 1% poloxamer to 2.60±0.11 g in the animals treated with Exosurf® and vitamin E. Dry weights ranged from 0.31 ± 0.01 g for animals (given Infasurf to 0.42 ± 0.03 g) in animals treated with Exosurf® (40.5 mg

Table 14. Effects of surfactant replacement on lung weights 6 hours after phosgene exposure. Animals were exposed to 40.5 ppm phosgene for 10 minutes and treated with either saline (sham treated) or Exosurf® by intratracheal instillation as indicated (See Table 13 legend for details of dose and volume for the respective treatment conditions).

Treatment	N	Wet Weight Mean ± SE	Wet Weight t-test	Dry Weight Mean ± SE	Dry Weight t-test	Ratio Mean ± SE
Sham treated	48	2.33 ± 0.08		0.37 ± 0.01		6.32 ± 0.11
Exosurf (Instilled)						
Low Dose	16	2.35 ± 0.13	NS	0.36 ± 0.01	NS	6.49 ± 0.20
High Dose	9	2.31 ± 0.20	NS	0.35 ± 0.02	NS	6.47 ± 0.31
Low concentration	14	2.41 ± 0.10	NS	0.39 ± 0.02	NS	6.28 ± 0.17
Standard concentration	33	2.44 ± 0.08	NS	0.42 ± 0.03	NS	6.12 ± 0.22
High concentration	18	2.19 ± 0.11	NS	0.36 ± 0.02	NS	6.16 ± 0.25
Pretreatment	16	2.23 ± 0.10	NS	0.37 ± 0.01	NS	6.05 ± 0.21
Exosurf (Instilled) + Vitamin E added	19	2.60 ± 0.11	p<.05	0.39 ± 0.01	NS	6.73 ± 0.13
1% Poloxamer 188 added	19	2.07 ± 0.10	p<.05	0.33 ± 0.01	<.05	6.30 ± 0.17
Exosurf (Aerosol) 1 hour	20	2.42 ± 0.11	NS	0.37 ± 0.02	NS	6.54 ± 0.13
Other Surfactants/Agents						
Infasurf	10	2.11 ± 0.13	NS	0.31 ± 0.01	<.01	6.67 ± 0.24
Pure DPPC	8	2.19 ± 0.16	NS	0.33 ± 0.02	NS	6.56 ± 0.19
Tyloxapol	8	2.38 ± 0.17	NS	0.37 ± 0.02	NS	6.43 ± 0.23

NS = Not Significant

DPPC, 40.5 mg/ml). Wet / dry ratio ranged from 6.12 ± 0.22 in animals treated with Exosurf® (40.5 mg DPPC, 40.5 mg/ml) to 6.68 ± 0.20 in animals given Exosurf® + Vitamin E.

All of the treatment group weights were significantly increased over those observed prior to phosgene exposure (wet weight 1.27 ± 0.13 g; dry weight 0.26 ± 0.01 g; wet/dry ratio 4.88 ± 0.10) (t-test; $p \leq .01$). Only two of the treatment group wet weights were significantly different from those observed in sham-treated, phosgene-exposed rats (wet weight: 2.33 ± 0.08 g). Rats treated with Exosurf + Vitamin E had significantly higher wet weights than sham treated animals; rats treated with Exosurf + 1% Poloxamer had a significantly lower wet weight. Overall, there was little evidence to suggest that SRT attenuates the major outpouring of edema fluid, that occurs during the first 6 hours after exposure to an $LCt_{50,10,24}$ dosage of phosgene.

Effects of SRT on lung weights 24 hours after exposure to phosgene

By 24 hours after exposure, we did observe a beneficial effect of SRT on pulmonary edema in some but not all of the SRT treatments as indicated in Table 15. In general,animals given treatments that favorably influenced 24 hour mortality were found to have less tissue edema at 24 hours than those given treatments which did reduce phosgene mortality. This relationship is demonstrated in Figure 11, a scatter plot comparing mortality and lung wet weight at 24 hours for the treatment groups listed in Table 15. There was a strong correlation (correlation coefficient = 0.736) between 24-hour mortality and 24-hour wet weight for the respective treatment groups.

Effects of SRT on epithelial lining fluid composition 4 hours after exposure to phosgene

The effects of SRT on the airway/alveolar epithelial lining fluid composition are summarized in Table 16. SRT had little if any effect on the dramatic changes in epithelial lining fluid composition which occur soon after phosgene inhalation.

Four hours after exposure, BALF protein concentration ranged from 5.9 ± 0.8 to 8.0 ± 0.8 mg/ml for the various SRT treatment groups. None of these values exceeded the average protein concentration observed in sham-treated rats (8.2 ± 0.6 mg/ml). However, none of the treatment groups was statistically different from either the sham-treated rats or untreated, exposed rats.

Figure 11

Correlation of mortality effects with 24 hour tissue edema for SRT treatment groups

Scatter plot of mortality effects versus the degree of tissue edema observed 24 hours after exposure to 40.5 ppm phosgene for 10 minutes. Each point represents one of the treatment groups listed in Table 15.

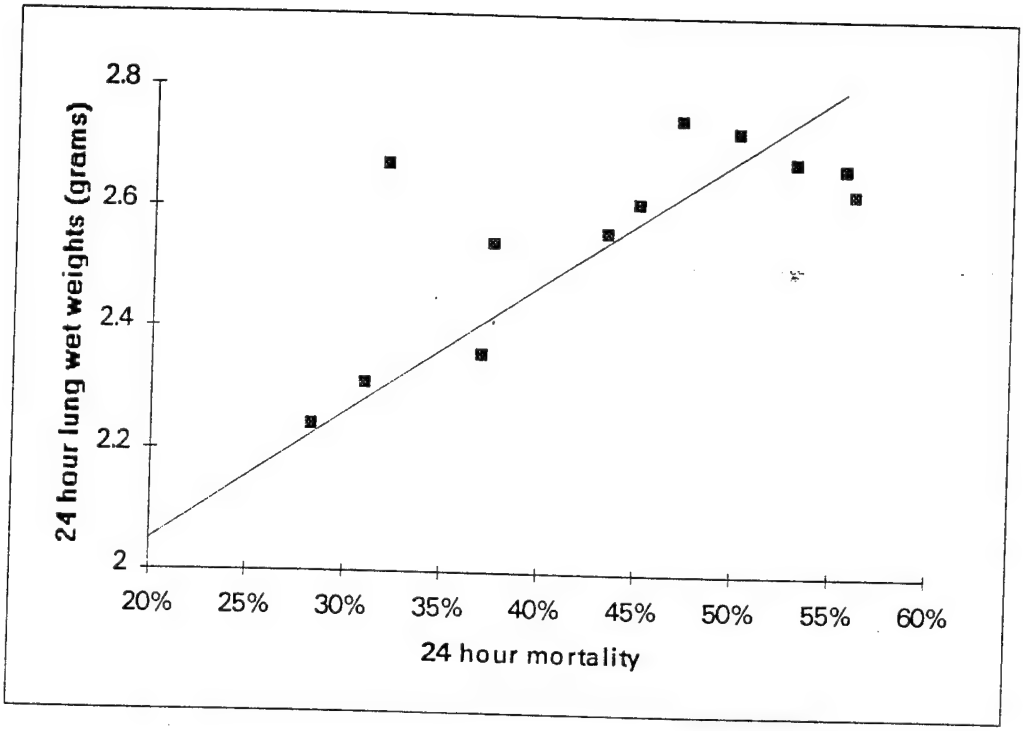


Table 15. Effects of surfactant replacement on lung weights 24 hours after phosgene exposure. Animals were exposed to 40.5 ppm phosgene for 10 minutes and treated with either saline (sham treated) or Exosurf® by intratracheal instillation as indicated (See Table 13 legend for details of dose and volume for the respective treatment conditions).

Treatment	N	Wet Weight Mean ± SE	Wet Weight t-test	Dry Weight Mean ± SE	Dry Weight t-test	Ratio Mean ± SE	Ratio t-test
Sham treated	67	2.68 ± 0.05		0.52 ± 0.01		5.15± 0.07	
Exosurf (Instilled)							
Low concentration	9	2,63 ± 0.12	NS	0.53 ± 0.03	NS	5.01 ± 0.21	
Standard concentration	14	2.36 ± 0.07	NS	0.48 ± 0.01	p<.05	4.94 ± 0.11	
High concentration	16	2.31 ± 0.08	p<.05	0.47 ± 0.02	p<.05	4.91 ± 0.05	p<.05
Exosurf (Instilled) + Vitamin E added	25	2,54 ± 0.09	NS	0.51 ± 0.02	NS	4.95 ± 0.08	p<.05
1% Poloxamer 188 added	14	2.24 ± 0.10	p<.01	0.44 ± 0.02	p<.05	5.17 ± 0.12	NS
Exosurf (Aerosol)							
Sham treated	22	2.67 ± 0.12		0.57 ± 0.04		5.17 ± 0.17	
2 hour treatment	11	2.61± 0.14	NS	0.51 ± 0.03	NS	5.15 ± 0.19	NS
4 hour treatment	14	2.75 ± 0.12	NS	0.54 ± 0.03	NS	5.17 ± 0.20	NS
Other Surfactants/Agents							
Infasurf	18	2.67 ± 0.11	NS	0.51 ± 0.02	NS	5.21 ± 0.13	NS
PureDPPC	17	2.56 ± 0.16	NS	0.51 ± 0.02	NS	4.98 ± 0.13	NS
Tyloxapol	14	2.73 ± 0.21	NS	0.52 ± 0.03	NS	5.15 ± 0.17	NS

Treatment effects analyzed by unpaired t-test vs. sham treated group. NS = Not Significant

Table 16. Effects of surfactant replacement on BALF composition 4 hours after phosgene exposure. Animals were exposed to 40.5 ppm phosgene for 10 minutes and treated with either saline (sham treated) or Exosurf® by intratracheal instillation as indicated (See Table 13 legend for details of dose and volume for the respective treatment conditions).

Treatment	N	Protein (mg/ml)	LDH activity (nmol NADH oxidized/ml)
Sham treated	36	8.2 ± 0.6	91 ± 25
Low Dose	18	5.9 ± 0.8	85 ± 45
Standard Dose	16	7.5 ± 0.6	86 ± 30
High Dose	16	8.0 ± 0.8	86 ± 29
Exosurf+ 1% Poloxamer	20	6.2 ± 0.4	89 ± 39
Exosurf +Vitamin E	17	8.0 ± 0.7	68 ± 26
Exosurf Pretreatment	17	6.4 ± 0.9	64 ± 20‡

Treatment effects analyzed by unpaired t-test vs. sham treated group. Significance levels: ‡$p \leq 0.05$

We did find a statistically significant effect of SRT on BALF LDH activity in animals treated with Exosurf® before exposure to phosgene (64 ± 5 vs. the sham-treated average of 91 ± 4 nmol NADH oxidized/min, $p \leq .05$). Apparently, pretreating with surfactant attenuates the cytotoxic effects of the gas on the airway lining cells. This same benefit was not afforded if treatment was given after the exposure.

We also observed an effect of treatment on BALF cytokine levels. Phosgene exposure had a major effect on the airway lining fluid IL-6 levels. Within the first 15 minutes after exposure,IL-6 was found to have increased from a pre-exposure value of 2680±233 to 3450±218 pg IL-6 per ml. By six hours after exposure, IL-6 increased to 4411±1104 pg per ml in the absence of treatment with SRT and to 6156±798 pg per ml in exposed rats that were treated with Exosurf + Poloxamer (40.5 mg DPPC,81 mg/ml,1% Poloxamer w/v) immediately after exposure.By twenty four hours after exposure,IL-6 levels again rose to 30531±6280 pg per mlwithout SRTand to 57002±2846 pg per ml in Exosurf-Poloxamer treated rats. The effect of treatment with SRT was not however statistically significant at either 6 or 24 hours after exposure and treatment.

We observed a small but significant effect of exposure on BALF fibronectin observed immediately after exposure. Within the first 15 minutes after exposure, fibronectin recovery from the airways increased from a pre-exposure value of 0.9±.3 to 1.8±0.3 ug per ml BALF. Over the next six hours, fibronectin levels continued to rise to 41.6±5.1 ug per ml in untreated rats and to 47.0±6.8 ug per ml in Exosurf-Poloxamer treated (40.5 mgDPPC, 81 mg/ml,1% Poloxamer w/v) rats. A significant effect of treatment was observed over the next 16 hours. Fibronectin continued to accumulate in the airways in untreated (24 hour BALF recovery = 86±6.3 ug per ml) but not Exosurf-Poloxamer treated rats (44.5±8.0 ug per ml BALF).

Effects of SRT on PS system after exposure to phosgene

Treatment with Exosurf® immediately after phosgene exposure had no effect on the amount of phospholipid recovered in the PS fraction of the BALF 4 hours after phosgene exposure (Table 17). Treatment did have a beneficial effect on the surface activity of the PS isolated from these animals. For example, PS isolated from animals treated with Exosurf® (13.5, 40.5 or 135 mg

Table 17. Effects of surfactant replacement on PS 4 hours after phosgene exposure. Animals were exposed to 40.5 ppm phosgene for 10 minutes and then treated with either saline (sham treated) or Exosurf® by intratracheal instillation as indicated. Lung lavages were performed 4 hours after exposure/treatment. PS was isolated from the BALF by centrifugation and suspended in BALF to a concentration of 2 mg phospholipid/ml. Surface tension was measured using a pulsating bubble surfactometer by the method of Enhorning [29] at minimal bubble radius after 100 bubble pulsations (5 minutes).

Treatment	Exosurf® Dose (mg DPPC)	PS Phospholipid (mg)	Surface Tension (mN/m)
Sham treated	0	0.16 ± .02 (N=7)	22.5 ± 3.2 (N=7)
Low Dose	13.5	0.31 ± .06 (N=7)	8.5 ± 6.6 (N=3)
Standard Dose	40.5	0.22 ± .03 (N=7)	7.0 ± 4.9 (N=2)
High Dose	135	0.22 ± 0.2 (N=5)	6.5 ± 4.5 (N=4)
All Exosurf® treated rats (low+ standard+high dose)			7.3 ± 2.8 (N=9)‡

Treatment effects analyzed by unpaired t-test vs. sham treated group. Significance levels: ‡$p \leq 0.05$

DPPC) and suspended in protein-rich BALF was significantly more effective in lowering the surface tension of a 0.275-µl pulsating bubble than PS isolated from sham-treated, phosgene exposed rats (Table 17).

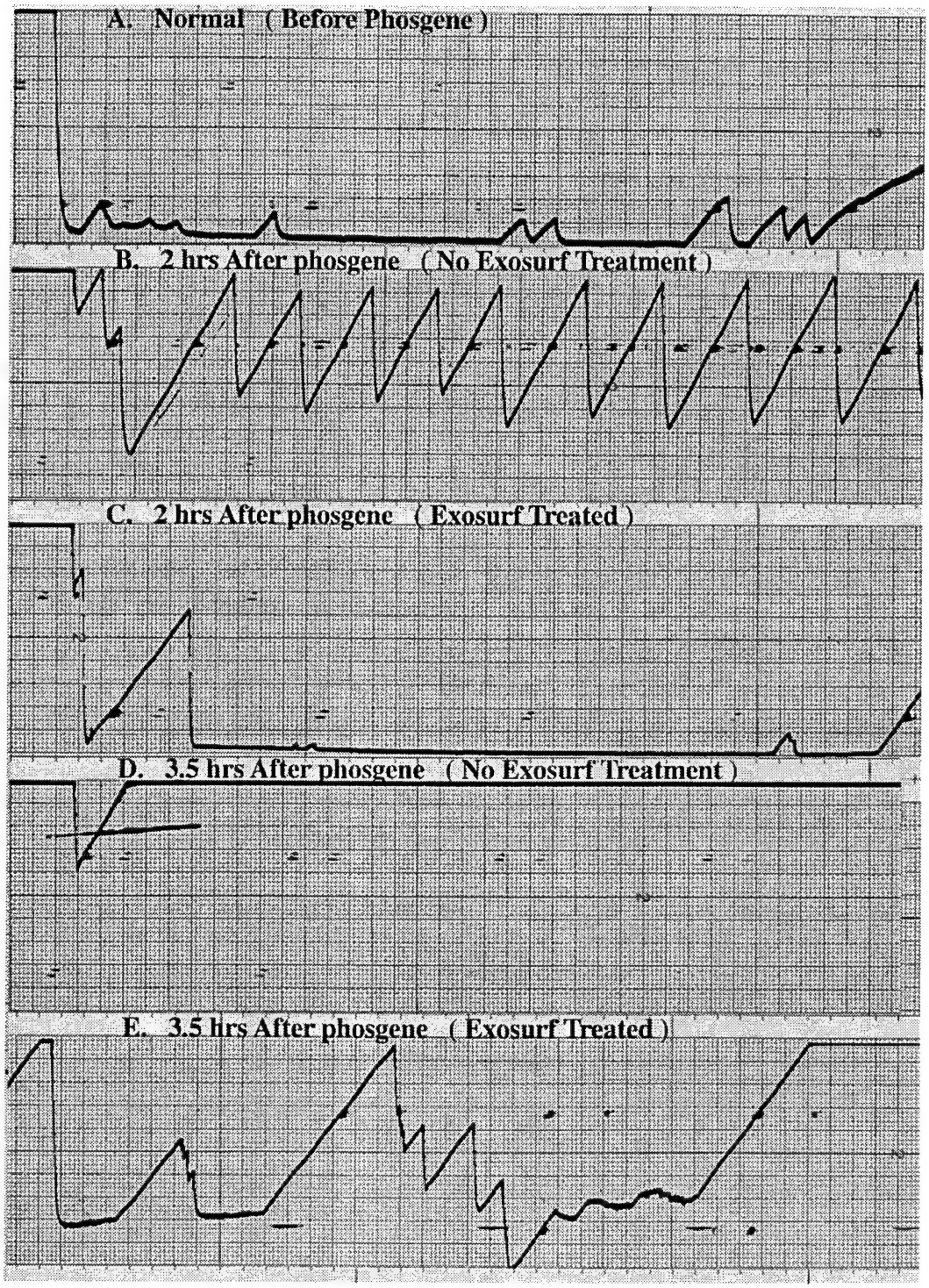
An *in situ* assessment of PS was accomplished by measuring the resistance that meets a steady flow of air through the conducting airways of extirpated lungs [Fig.12]. A tracing of pressure [Panel A, Fig. 12] showed a drop in resistance or pressure due to the flow of air extruding the columns of liquid blocking the lumen of the airways in a control or normal rat lung. After extrusion of the liquid columns that can block the lumens, the airways will remain open due to well-functioning pulmonary surfactant preventing the liquid columns from returning. In Panel A where the pulmonary surfactant is normal, the long period of low steady pressure indicated airway patency. Two hours post-exposure to phosgene [Panel B] the serrated tracing was consistent with altered or damaged surfactant which allowed the liquid columns to form in the lumens and augment resistance to airflow. In Panel C it can be seen in the rats treated with Exosurf + 1% Poloxamer were near normal in their ability to maintain patency in the conducting airways two hours after exposure to phosgene. The pulmonary surfactant was dysfunctional 3.5 hours post-exposure to phosgene [Panel D] and as shown in Panel E the Exosurf + 1% Poloxamer was much less effective in maintaining low airway resistance than at two hours post-exposure to phosgene.

It has previously been demonstrated that the inhibitory effects of fibrin, one of the suspected inhibitors of surfactant function in the adult respiratory distress syndrome, can be antagonized by the synthetic surfactant, Poloxamer 188 [37]. We performed a series of studies to assess the effect of Poloxamer 188 on PS function and determine whether Poloxamer 188 could be used *in vivo* to prevent PS inhibition by plasma proteins.

We found that the pulmonary surfactant isolated from the phosgene poisoned animals 4 hours after exposure and suspended in saline rapidly reduced surface tension during bubble compression to an average 2 mN/m after 5 minutes, well within the range found with surfactant isolated from normal rats and tested under identical circumstances. In contrast, we found the surface properties of the pulmonary surfactant isolated from the exposed animals were markedly

Figure 12
Effect of SRT on Airway Patency

Animals were exposed to either 40.5 ppm phosgene or air for 10 minutes, then given 0.5 ml of either Exosurf® + 1% Poloxamer or 0.9% saline by intratracheal instillation. Airway patency was examined at intervals indicated.



abnormal when we mixed the surfactant with the protein rich BAL fluid throughout the compression/expansion cycle (see Figure 13). Admixed with BAL fluid, surface tension at minimum bubble volume after 5 minutes was on the average 22 mN/m. These findings again support the conclusion that the epithelial lining in the exposed rat contains one or more surfactant inhibitors, e.g., fibrin, and that it is the leakage of inhibitors into the alveolar lining fluid, not direct damage to the pulmonary surfactant itself, that accounts for the adverse effects of phosgene on pulmonary surfactant function.

We found that the addition of Poloxamer 188 in concentrations ranging from 1 to 5% to the BAL fluid isolated from exposed rats markedly reduced the inhibitory effects of the BAL fluid on the isolated surfactant (see Figure 14). Higher concentrations of poloxamer were found to have an adverse effect on the surfactant (see Figure 15) presumably as a consequence of concentrating at the air liquid interface and preventing access to the surface active molecules of the pulmonary surfactant. Lower concentrations (0.1 and 0.01% Poloxamer 188) were not effective (see Figure 16). These dose-respose effects of Poloxamer 188 in restoring PS function in the presence of BALF inhibitors are summarized in Figure 17.

Effect of SRT on lung function after exposure to phosgene

SRT was shown to transiently normalize several of the functional parameters that were adversely affected by phosgene inhalation (Figures 6 through 10). SRT reduced the breathing rate and increased the peak inspiratory and expiratory flow for up to 5 hours after exposure. All of these findings were made using free-breathing, awake rats in Fenn-type ventilated whole-body plethysmographs. The more direct measures of lung elasticity performed 5 hours after exposure indicated that any salutary effect SRT had on lung function was transient. With the single exception of a sustained beneficial effect of SRT treatment with Exosurf + 1% Poloxamer on lung compliance, none of the abnormal findings associated with phosgene inhalation, including decreased tidal volume, inspiratory and expiratory times, total lung and vital capacity, residual volume CO diffusion capacity or respiratory system compliance were significantly affected by SRT (Tables 11 and 12).

Figure 13
Effects of phosgene on surfactant function

Lung surfactant was isolated from phosgene exposed rats and suspended in either 0.9% saline (circles) or broncho-alveolar lavage fluid from a phosgene exposed rat (squares). Surfactant function was assessed using the pulsating bubble technique of Enhorning [Reference 29].

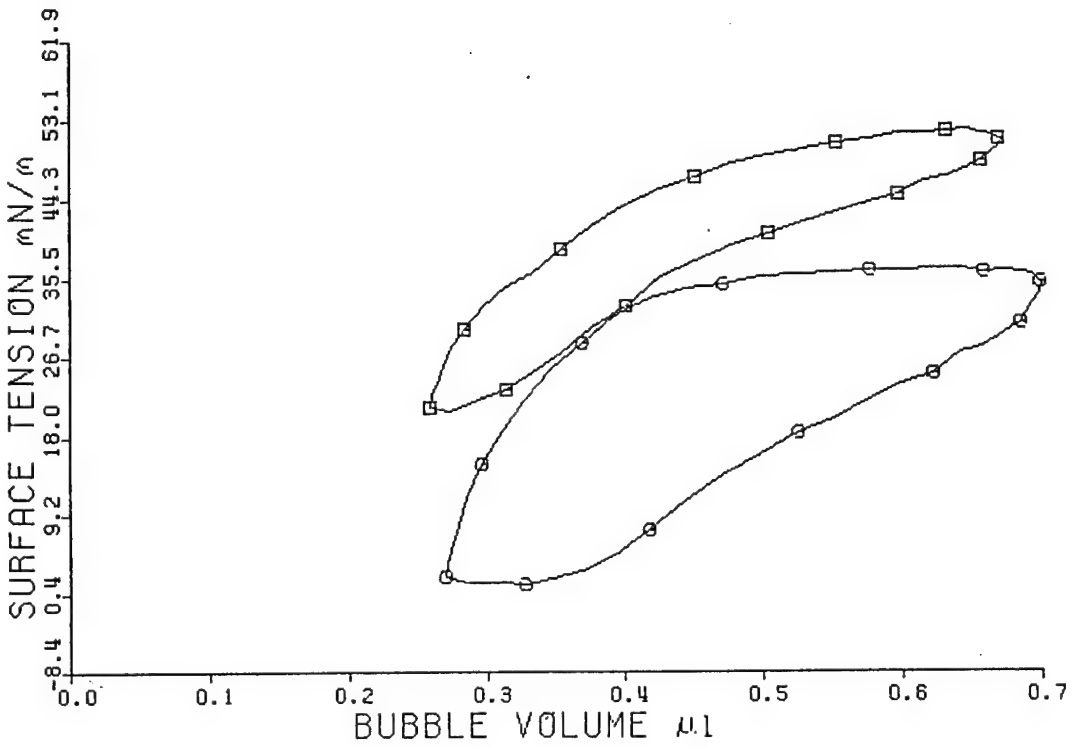


Figure 14
Effects of 1% Poloxamer 188 on surfactant function

Lung surfactant was isolated from phosgene exposed rats and suspended in broncho-alveolar lavage fluid from an exposed rat with (squares) or without (circles) added Poloxamer 188 (1% w/v). Surfactant function was assessed using the pulsating bubble technique of Enhorning [Reference 29].

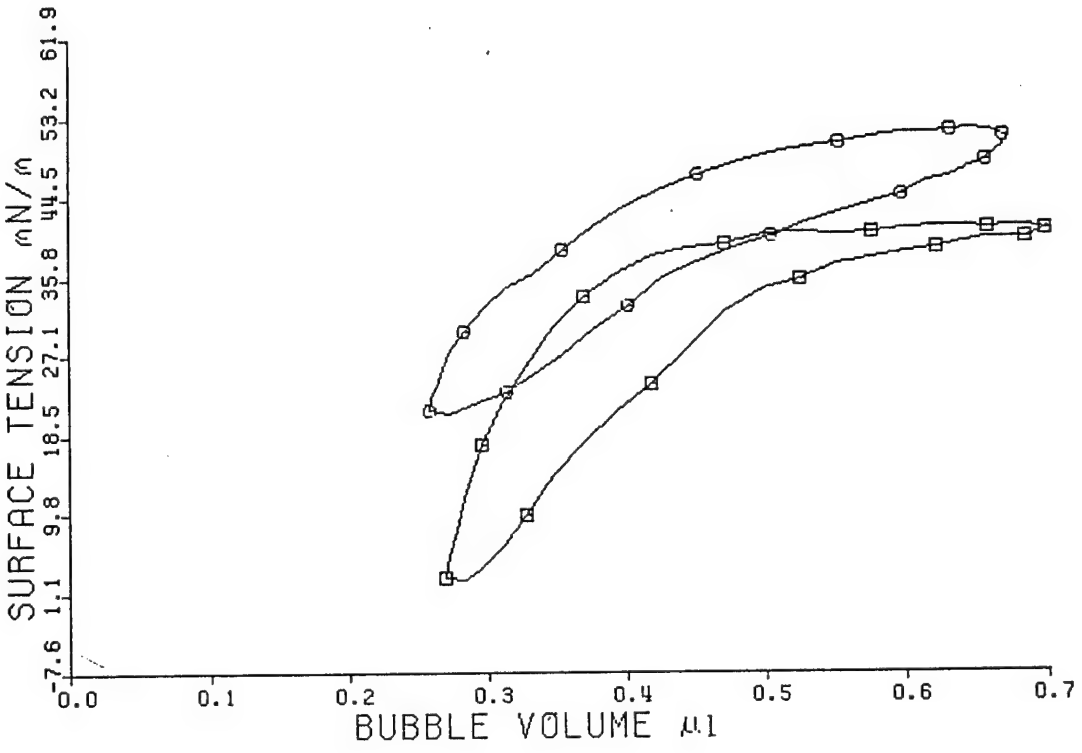


Figure 15
Effects of 10% Poloxamer 188 on surfactant function
Lung surfactant was isolated from phosgene exposed rats and suspended in broncho-alveolar lavage fluid from an exposed rat with (squares) or without (circles) added Poloxamer 188 (10% w/v). Surfactant function was assessed using the pulsating bubble technique of Enhorning [Reference 29].

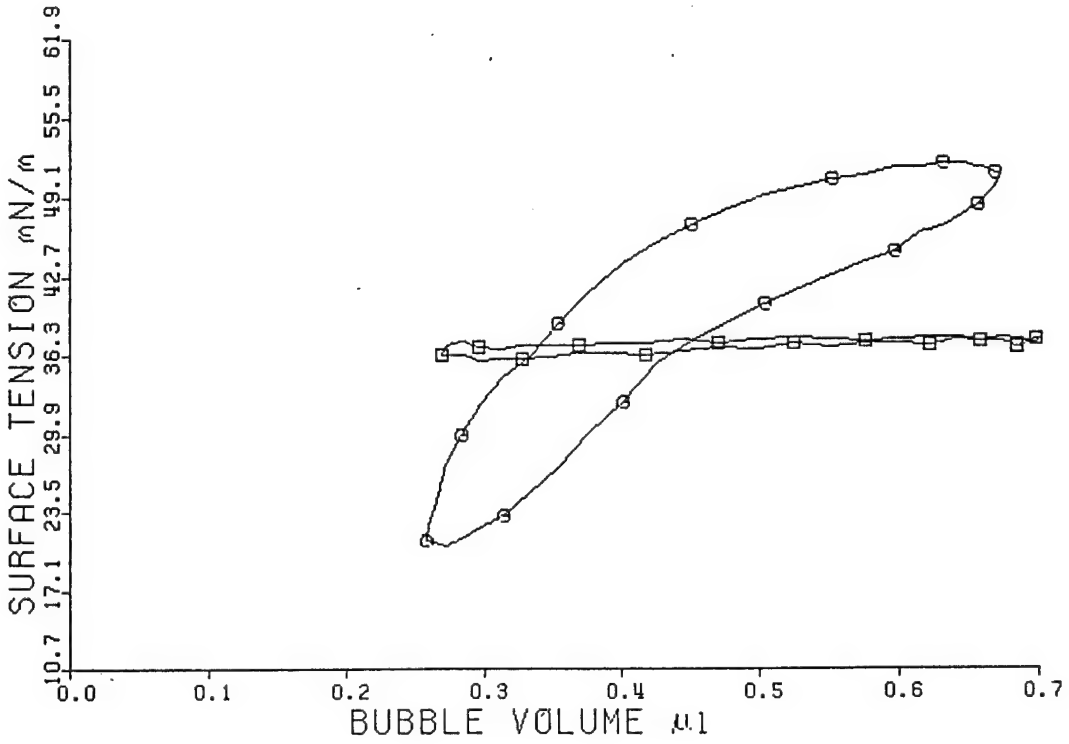


Figure 16
Effects of 0.1% Poloxamer 188 on surfactant function

Lung surfactant was isolated from phosgene exposed rats and suspended in broncho-alveolar lavage fluid from an exposed rat with (squares) or without (circles) added Poloxamer 188 (0.1% w/v). Surfactant function was assessed using the pulsating bubble technique of Enhorning [Reference 29].

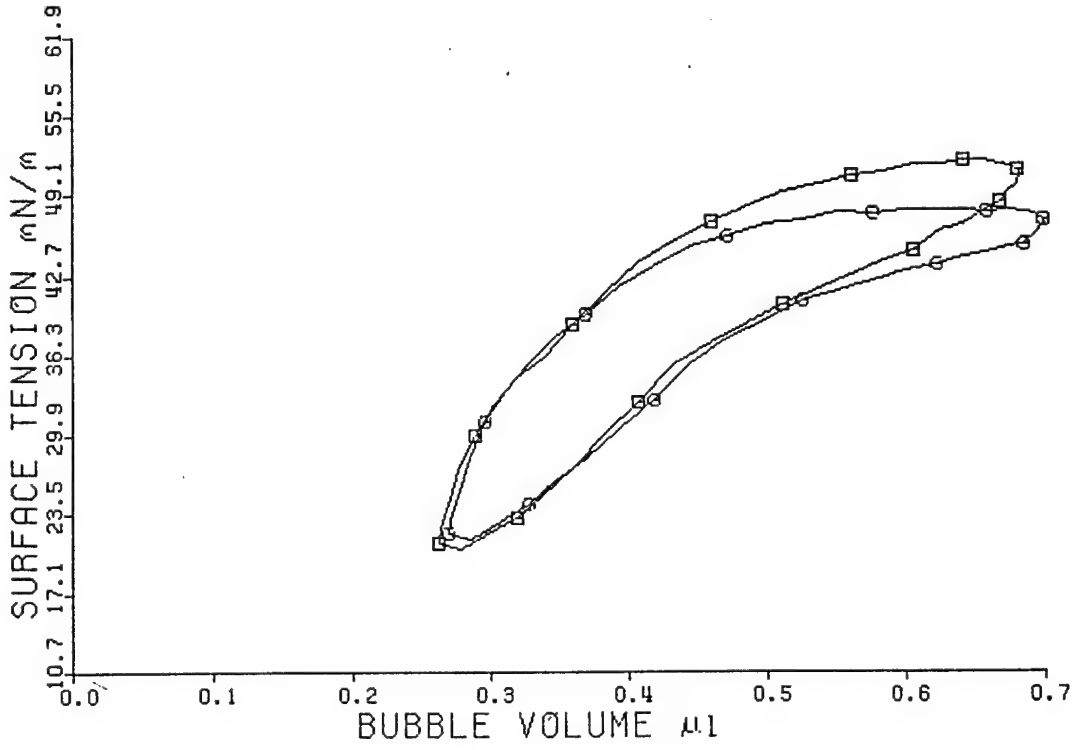
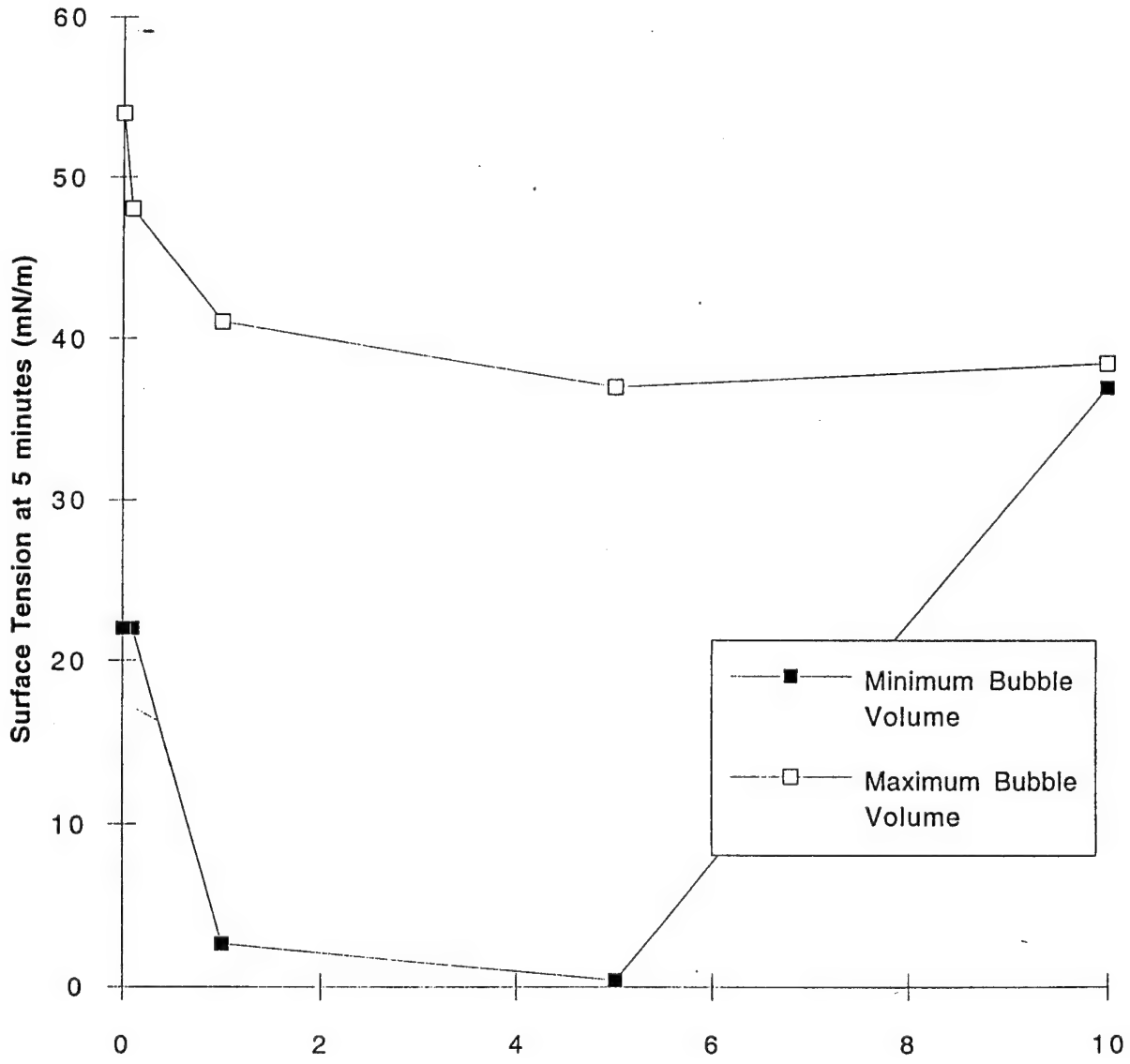


Figure 17
Effects of Poloxamer 188 on surfactant function

Lung surfactant was isolated from phosgene exposed rats and suspended in broncho-alveolar lavage fluid from an exposed rat. Poloxamer 188 was added to the suspension at the indicated concentration immediately before assessing surfactant function using the pulsating bubble technique of Enhorning [Reference 29]. Surface tension at minimum and maximum bubble volume was measured after 100 oscillations (5 minutes).



Effect of SRT on lung morphology 24 hours after exposure to phosgene

There were no remarkable differences between animals treated with Exosurf® and sham-treated animals as sacrificed 24 hours after exposure to phosgene. The treated lungs again were found to have diffuse edema with alveolar septal thickening, alveolar flooding and atelectasis and hemorrhage. Perivascular edema was again apparent throughout the tissue. PMN leukocytes were found in all the sections, primarily in association with the edema fluid.

Effect of Surfactant Treatment on the Phosgene $LCt_{50,10,24}$

The effect of surfactant treatment on the $LCt_{50,10,24}$ dosage for phosgene was determined in a set of 100 rats exposed for 10 minutes to one of the five following phosgene concentrations: 20, 30, 40, 55 or 70 ppm. Immediately after exposure, rats were treated with 0.5 ml of Exosurf + Poloxamer (40.5 mg DPPC + 1% Poloxamer 188) by intratracheal instillation. Ninety-eight of the rats survived the instillation; two died during treatment (one exposed to 40 ppm and one to 55 ppm phosgene) and were not included in the determination of the $LCt_{50,10,24}$. The results of these studies are presented in Table 18. The estimated $LCt_{50,10,24}$ for surfactant treated rats was 450 ppm·minutes.

Table 18. LCT_{50} determination, Surfactant Treated Rats: Phosgene 10-minute exposures, 24-hour survival. Immediately after exposure, rats were individually anesthetized with halothane and given 0.5 ml of Exosurf + Poloxamer (40.5 mg DPPC + 1% Poloxamer 188) by intratracheal instillation.

Phosgene Dose (ppm·min)	N	24-hour Mortality
200	10	0%
300	10	10%
400	39	28%
550	29	86%
700	10	90%

N=number of phosgene exposed rats surviving SRT and recovering from halothane.

Discussion

Up to now the role of the PS system in phosgene intoxication has been largely ignored with a few notable exceptions [15, 16, 25]. The present findings extend these earlier studies, confirming our hypothesis that phosgene inhalation adversely affects PS surface activity. Our studies further suggest that surfactant replacement may be an effective countermeasure for the effects of this toxic gas on the PS system and for reducing mortality from phosgene exposure.

Effect of phosgene exposure on the PS system

In normal lung, PS serves to prevent collapse of alveoli and preserve patency of terminal bronchioles by reducing the surface tension of the air-epithelial lining fluid interface. Our observations confirm that phosgene exposure adversely affects both of these PS functions. Numerous factors may contribute to the decline in PS function. Cytotoxicity may play a role. Damage to alveolar cells may decrease the synthesis and recycling of PS [15, 16]. We did observe an increase in LDH activity in the BALF recovered from phosgene exposed rats. The presence of LDH, a cytosolic enzyme, in the epithelial lining fluid suggests that phosgene did damage epithelial lining cells. Further studies will be required to determine whether the damage extended to pneumocyte type II cells, the site of PS metabolism. In any case, we did not discern any increase in BALF LDH activity four hours after phosgene exposure, i.e., at a time that we were able to detect abnormal PS surface function. Therefore, it appears unlikely that the cytotoxic effects of phosgene account for all the effects of the toxin on the PS system. Rather, our studies suggest that the effects of phosgene on the PS system follow from changes in alveolar epithelial and endothelial permeability .

We did observe a temporal correlation between phosgene induced changes in BALF protein concentration and the deterioration of PS surface function. The microvascular permeability changes triggered by phosgene introduces numerous proteins, e.g., albumin and fibrin, some of which bind phospholipids and may alter the normal association of DPPC with neutral lipids and hydrophobic proteins to form the requisite lipoprotein complex [21]. Interestingly, we could only detect

abnormal surface activity in response to phosgene exposure if the PS was suspended in BALF, not in saline. The possibility that BALF from phosgene exposed rats contains one or more components which interfere with the surface activity of PS is further supported by our observation that PS isolated from normal rats (no phosgene) and suspended in BALF from a phosgene exposed rat also exhibits abnormal surface activity. The inactivation of PS with BALF from exposed animals may be due to a specific inhibitor, an overwhelming amount of BALF protein, or simply competition for the interface by the large number of components.

Other factors which might contribute to the deterioration of PS function following phosgene inhalation include direct oxidation of the neutral lipids and/or hydrophobic surfactant specific proteins which may interfere with their association with DPPC. Airway inflammation may also play a role. PMN leukocytes were consistently found during histological examination of phosgene exposed lung sections. Activated inflammatory cells are known to release enzymes and free radicals which have been shown to have an adverse effect on PS [21-23].

Role of SRT in ARDS

SRT has proven to be very beneficial in the treatment of neonatal respiratory distress syndrome (RDS), with improvement in physiological parameters and mortality of average and very low birthweight babies. Both Exosurf and Survanta surfactants are approved by the FDA for rescue and prophalyxsis treatment of RDS. RDS in newborns occurs because the premature lung lacks natural surfactant due to a lack of initiation of the biosynthetic pathways.

ARDS has many physiological similarities to RDS, including a deficiency of and abnormal composition of pulmonary surfactant [40]. However, the disease differs from the neonatal counterpart in that it develops due to inhibition of biosynthetic pathways and/or inactivation of preformed surfactant by products leaking from the plasma. ARDS may develop after sepsis, acute trauma, major burns, long bone breaks, and pancreatitis. While some of these predisposing factors may have specific components that act on the lung directly such as endotoxin and fat

emboli, they all share a commonality of altered levels of circulating cytokines which may cause leakage of the pulmonary vasculature.

Since natural surfactant levels are altered in ARDS, SRT represents a logical treatment regimen for the disease. SRT improves lung physiology and gas exchange in several animal models of ARDS, including oxygen toxicity, lavage depletion, drowning, endotoxin, and phosgene poisoning. Both Exosurf and Survanta have been used to treat ARDS in controlled clinical trials.

Exosurf has been administered to sepsis patients by nebulization using a complicated proprietary breath actuated nebulizer coupled to a ventilator. In two small trials with a total of 100 patients [41] Exosurf was administered for 12 or 24 hours per day for 5 days using DPPC concentrations of 13.5, 40.5 or 81.0 mg/ml:

Number	mg DPPC/ml	Hr/day aerosolized	Mortality %
17	placebo	12 & 24 combined	47
16	placebo	12	50
17	13.5	12	41
17	13.5	24	35
16	40.5	24	25
17	81	24	35

The maximum difference in mortality incidence of 25% occurred with aerosolization of 40.5 mg/ml DPPC for 24 hours. Greater amounts of Exosurf, i.e. 81 mg/ml were less beneficial. All patient groups had an Apache II score of 16-18. These data prompted a larger study [42] of 725 patients, although mortality data have only been reported for the first 498:

Treatment	Overall	Apache III Score		
	Mortality	0 - 45	46 - 90	> 90
Surfactant	41%	29%	35%	73%
Placebo	41%	18%	38%	73%

In the entire study group, surfactant treatment did not alter 30 day survival. This prompted the Burroughs Wellcome Co. to discontinue further trials of Exosurf for treatment of ARDS. It should be pointed out that the overall mortality of 41% is lower than the historic norm for this patient population. These data are complicated by the fact that newer antibiotics, ventilator management, and other changes in treatment management improved while this trial was in progress. However, in the less severe patients, i.e., those with Apache III scores of 0 - 45, Exosurf treatment did reduce mortality. Thus treatment with aerosolized Exosurf may be most beneficial in the less severe patients. This is reasonable because Exosurf is only treating the respiratory symptoms and improving ventilation, it does not treat the underlying sepsis or provide direct benefit to other organs that may be affected as multiorgan failure develops with a high Apache score. In both studies Exosurf improved arterial oxygenation.

Survanta has been studied less extensively with a single report of 59 patients of mixed etiology, i.e., sepsis, trauma, gastric aspiration and multiple transfusions [43]:

	Standard Therapy (n=16)	50 mg PL/kg max 8 doses (n=8)	100 mg PL/kg max 4 doses (n=16)	100 mg PL/kg max 8 doses (n=19)
FiO2	-0.14± 0.06	-0.05±0.11	-0.35±0.06	-0.06±0.08
PEEP (cm H20)	-3.2±1.6	-3.6±1.6	-7.4±2.1	-1.6±1.1
PaO2/FiO2	23.0±24.8	30.7±34.6	63.5±22.3	36.9±20.9
Mortality	43.8	50	17.6	21.1

The maximum improvement in physiology and mortality occurred with 4 doses of 100 mg/kg of DPPC, with 8 doses having less effect. In these trials the Survanta surfactant was given as a liquid bolus via an endotracheal tube similar to the way the drug is given to neonates. Survanta has been studied less extensively than Exosurf because the drug is more difficult to prepare, the starting material for production is a cow lung extract. Commercial use of Survanta to treat ARDS would require a revamping of the slaughterhouse industry because the lungs are not an organ that is presently in high demand.

Thus, both Exosurf and Survanta surfactants have been shown to reduce mortality in selected small groups of patients. However, many questions remain unanswered because of the small sample populations and the way in which the studies were conducted.

Both drugs suggest that there is an optimum dose and that too much surfactant may be less beneficial. With both drugs the volume of surfactant administered was several fold greater than the normal endogenous supply of surfactant. The present phosgene studies also suggest that there is an optimum dose of surfactant which may vary according to how the drug is formulated, or whether it is presented as a bolus or as an aerosol. A great deal of work will be required to define the optimum dose.

The Exosurf data suggest that less severe patients respond better. This may be a real observation or simply mean that the optimum dose varies with the severity of the disease. Phase II trials of Exosurf were conducted without careful animal studies of the nebulizer system. Little information is known regarding the dose of Exosurf administered or retained by the patients. The nebulizer setup was technically difficult and no information was reported as to optimum particle size.

Both the clinical trials with ARDS patients and the present phosgene exposure studies suggest both that Exosurf and Survanta are effective is reducing mortality while improving physiology and that both aerosol and liquid bolus administration are effective. This is a surprising observation given the wide variation in techniques and strongly argues that SRT has a role in treating ARDS.

The aerosol administration of surfactant is technically more difficult and wastes more drug but is less invasive to the patient. Animal studies show that aerosolized drug is deposited mainly in normal alveoli rather than diseased areas and that the physiological improvement s are due to greater shunting of blood flow to the normal areas [41]. A similar observation has been made regarding the beneficial effects of NO on the pulmonary circulation. However, large volumes of surfactant given by liquid bolus open up the diseased alveoli. Giving NO inhalation after a liquid bolus of surfactant produced a synergistic improvement in lung function in a lavage depletion

model of ARDS [44]. Combining liquid bolus and aerosol techniques to administer surfactant to ARDS patients has not been tried.

Many therapeutic agents have been tested in ARDS including various anti-inflamatory agents and anti-endotoxin fragments with limited and varying success. SRT has produced the largest improvements in lung function and greatest decreases in mortality. Yet SRT may never attain commercial viability unless there is sufficient research by independent investigators or a new company enters the market. Too many questions pertaining to dosing and route of administration remain unanswered. Burroughs Wellcome Co. has dropped development of an ARDS indication for Exosurf. Many investigators criticized this decision as premature based on inadequate information. The Survanta product marketed by Ross is expensive to produce and in too short of a supply for the large scale trials needed to sufficiently resolve the unanswered questions.

ARDS is a complicated and variable disease that is but one component of a systemic insult on several organ systems. SRT needs further investigation to more fully understand the dosage profile and then the SRT therapy combined with other treatment modalities such as anti-inflamatory agents to normalize the cytokine levels that are causing the vascular leakage.

Effectiveness of SRT as a countermeasure for phosgene poisoning

The present studies demonstrate that SRT significantly decreases the 24-hour mortality from phosgene exposure. We observed about the same degree of attenuation of the lethal effects of exposure using three different surfactant replacements: Exosurf®, Exosurf® supplemented with Poloxamer 188, and Infasurf®. SRT pretreatment protected the airways from the cytotoxic effects of phosgene as indicated by a reduced amount LDH recovered in BALF. SRT had no effect on the massive edema that occured at 4-6 hours after exposure to phosgene, nor did it have any sustained effect on lung function.

We had hypothesized that by supplementing the PS following phosgene exposure we would alleviate or reduce the massive edema which marks the onset of the clinically manifest stage of phosgene poisoning. It is generally accepted that by reducing interfacial surface tension at the

alveolar air-water interface natural PS and presumably instilled exogenous surfactant increases alveolar stability and prevents alveolar collapse at each end expiratory volume. Pattle [12] suggested that the extremely low surface tensions in the alveolus would counter the hydrostatic pressure in the alveolar capillaries promoting net water flow out of the alveolus. Pattle's hypothesis was for a normal lung with an intact epithelium and endothelium. The present studies suggest that the alveolar damage, whether endothelial or epithelial, creates holes large enough that the principles of the Starling equation no longer apply. Consequently, SRT does not prevent the massive edema at 4-6 hours.

In spite of this, SRT does significantly improve mortality whether given immediately after exposure, prior to exposure or even six hours after exposure. The effectiveness of Exosurf® in reducing mortality was apparent only at a dosage of 40.5 mg DPPC. We did not see an effect at either 13.5 or 135 mg DPPC. It is possible that higher concentrations are not well tolerated by the phosgene damaged lung, although we found no evidence of adverse reaction to the drug in normal rats apart from a transient inflammatory response. Infasurf® (20 mg DPPC) provided about the same degree of protection from the lethal effects of phosgene as Exosurf® (40.5 mg DPPC). Neither drug had a discernible effect on edema or microvascular permeability as indicated by BALF protein concentration. Therefore, alternative explanations for how SRT improves survival are subject to speculation and deserving of further investigation, since it may shed significant new insights into why phosgene kills and/or how surfactants work in the lung.

In the neonatal lung where surfactant is absent there is little alveolar damage at birth. With breathing and constant alveolar collapse at EEV the epithelium is quickly damaged with edema and epithelial sloughing and formation of the characteristic hyaline membranes. SRT serves to stabilize the neonatal lung preventing epithelial damage and improving survival. In contrast, phosgene damages the epithelial lining in the presence of normal surfactant, leading to alveolar edema and possible surfactant alterations and/or inactivation. In this setting, SRT may be stabilizing partially flooded alveoli thereby preventing further damage and allowing the natural healing process to occur, for example over the first 24 hours after exposure in which the anti-edematogenic effects of SRT are

manifest. SRT may simply be serving as a source of DPPC, which might be used by recycling mechanisms to regenerate PS, the DPPC associating with other lipids and proteins in the alveolar lining fluid to reform PS. This possibility does not however seem likely, since we did not observe any significant attenuation of the lethal effects of phosgene by treating with pure DPPC. In interpreting this observation, it must be remembered that cetyl alcohol and tyloxopol serve to disperse and spread DPPC, which may affect the distribution of the instilled DPPC within the airways.

Is Exosurf® the preferred agent for SRT following phosgene inhalation?

Exosurf® and Infasurf® were found to be equally effective in attenuating the lethal effects of phosgene exposure. Exosurf® is a completely synthetic formulation containing no protein components. Infasurf® is an extract of natural PS and does contain protein. Natural PS is a mixture of phospholipids, neutral lipids and hydrophobic proteins that associate into at least 3 lipoprotein complexes of specific and reproducible densities. DPPC is the predominate component of PS and the component responsible for the surface tension lowering properties of PS. Although pure DPPC will produce an interfacial film capable of surface tensions approaching 0 mN/m when spread from organic solvents, DPPC spreads very poorly from aqueous suspension. The neutral lipids and proteins facilitate the spreading of DPPC in Infasurf®. In the synthetic surfactant Exosurf®, the hexadecanol facilitates the spreading of DPPC. Mixtures of DPPC and hexadecanol are an effective synthetic surfactant; however the mixture tends to form large aggregates that interfere with uniform dispersion throughout the lung. The non-ionic detergent tyloxapol was formulated in Exosurf® to create a suspension of particles that did not clog the small bronchioles and would disperse more uniformly.

Exosurf® can be produced much less expensively with a very long shelf life compared to protein-containing animal lavage extracts. Accordingly, Exosurf® would be the drug of choice since Exosurf® and Infasurf® are equally effective in survival studies. Upon the availability of a metered dose inhaler capable of delivering larger doses of drug, pretreatment would become a possibility since a whiff of phosgene would alert troops and preventative treatment could begin.

The U.S. Army and soldiers would benefit greatly from the above studies since Exosurf® can be delivered easily by nurses post-exposure in a field hospital. Delivery can be accomplished using inexpensive, disposable nebulizers (as we used) or by intubation and bolus injection as indicated by the studies above.

Surfactant replacement therapy as a countermeasure for lethal phosgene gas exposure: Future research efforts

SRT has been used very successfully to increase survival following exposure to LCt50 dosages of phosgene. No other treatment or antidote is currently available, thus SRT can be a very valuable treatment for field soldiers and others that are at hazard to receive accidental phosgene exposures. The success with SRT strongly indicates the need for further studies, and studies that will increase the effectiveness of SRT are described below.

In the first phase of continued studies, a second small animal model should be selected to demonstrate the effectiveness of SRT against phosgene in another animal. The hamster is a logical choice for this phase since it has been well studied in aerosol inhalation toxicology. For use in extrapolation to nose breathing people, hamsters present no significant problems associated with differences in regional deposition of inhaled aerosols.

In the second animal model extensive studies are needed to determine the optimal dose and means of delivery of exogenous surfactant. Aerosol and bolus injections need to be studied in normal and damaged lungs to determine that the optimal dose is delivered to the desired region of the lung. We have clearly shown that the highest concentration of surfactant in the instilled bolus does not yield the greatest survival in rats and that the volume of the bolus is extremely important. Also, aerosol studies in rats showed that the longest exposures were not the most effective. There is clearly an optimum dose and it needs to be determined in each species for each means of delivery. Aerosol studies should also be conducted in Rochester-type chambers (unrestrained) as well as in the Cannon chambers (nose-only, restrained).

The studies using this second animal model should be focused on determining the mechanism of protection by SRT. Attenuation of the lethal effects of phosgene by SRT does not appear to follow from an alleviation of the principal pathophysiological manifestations of phosgene poisoning, pulmonary edema and reduced mechanical compliance. Studies should be conducted on the effects of SRT on endogenous surfactant synthesis, cycling, and conversion to active forms as well as on cytokine and other mediator levels. These metabolic studies should be conducted concomitantly with survival studies to determine optimal dose and means of delivery of SRT.

These hamster studies should include an extensive series of lung function testing to determine the status of the lungs of animals at critical junctures following phosgene exposure. This testing should be done in Fenn boxes or in unrestrained, unanesthetized animals. Our studies on compliance were conducted on animals that were anesthetized and the phosgene-damaged lungs were tested under conditions that could have further damaged the lungs. We need to measure compliance, etc., using techniques that do not damage or stress lungs that are already damaged. These studies are necessary to fairly assess the effectiveness of SRT in protecting lung function in phosgene-damaged lungs.

Studies should be done in a primate model to more closely approximate human conditions. In larger animals it will be possible to intubate and determine the optimal volume, optimal concentration and number of boluses to be delivered. Aerosol studies are needed to determine the optimal particle size to be delivered, chamber concentration, length of aerosol exposure, and number of exposures to yield maximum survival after phosgene exposure.

These additional efforts should provide the USAMRDC with an adequate base of pre-clinical information upon which a final decision regarding the suitability of SRT as a treatment for US military personnel may be made.

Conclusions

- The $LCt_{50,10,24}$ for phosgene (10-minute exposure, 24-hour survival) is 405 ppm·min in untreated rats, and 450 ppm·min in rats receiving surfactant replacement immediately after exposure.
- Up to 50 mg of surfactant phospholipid can be safely instilled into the lungs of unexposed rats, eliciting at most a transient and reversible inflammatory response.
- Instilled surfactant tends to accumulate in the dependent regions of the lung. This is particularly evident when administering the drug in volumes of less than 3 ml/kg body weight. Dispersion of the instilled surfactant is improved when the animal was rotated between supine and prone positions immediately after treatment.
- The $LCt_{50,10,24}$ phosgene dosage causes massive edema, the major portion of which appears within the first 6 hours after exposure. Lung wet weights for animals surviving to 24 hours are more than double their normal value. Approximately 25% of the wet weight gains occurrs between 6 and 24 hours after exposure.
- The noninvasive nature of MRI combined with its sensitivity to pulmonary water content makes it an ideal tool for examining the temporal and spatial evolution of pulmonary edema following phosgene exposure. The lesions revealed by MRI following phosgene exposure can be distinguished before the massive outpouring of edema fluid. MRI studies demonstrated that lung damage following phosgene inhalation is diffuse, with areas of injury surrounded by normal tissue. Accordingly, MRI can be used to quantify the fraction of lung damaged by phosgene exposure.
- Phosgene has a profound effect on broncho-alveolar lavage fluid (BALF) composition. Within the first 4 hours after exposure, the BALF contains twice its normal amount of LDH, and BALF protein is increased by a factor of 40.

- Pulmonary surfactant isolated from phosgene-exposed lungs and suspended in BALF from exposed rats exhibits abnormal surface activity. PS isolated from normal (unexposed) rats and suspended in BALF from an exposed rat also exhibits abnormal surface activity.
- The adverse effects of phosgene on lung function is evident within minutes after exposure. Abnormal findings associated with phosgene includes increased frequency of breathing, decreased tidal volume, inspiratory and expiratory times, terminal bronchiole patency, total lung capacity, vital capacity, residual volume, CO diffusion and respiratory system compliance.
- The 24-hour mortality rate found in 189 sham-treated rats exposed to 405 ppm·min is 53%.
- Mortality rates for phosgene-exposed rats are significantly reduced by treatment with Exosurf®, Exosurf® supplemented with Poloxamer 188, or Infasurf®. SRT may be instituted by direct instillation into the airway or as an aerosol (particle size < 4 μm) either before exposure, immediately after exposure or up to 6 hours after exposure to attenuate the lethal effects of exposure. The 24-hour mortality rate observed under optimal treatment conditions in rats exposed to 405 ppm-min was 28%.
- The effect of SRT on mortality is strongly dose dependent. Optimal effects were observed at a dose of Exosurf® containing 40.5 mg DPPC. Loss of effectiveness was observed at both higher (135 mg DPPC) and lower (13.5 mg) doses.
- SRT does not attenuate the massive edema that occurs during the first 6 hours after exposure to the $LCt_{50,10,24}$ dosage of phosgene. A small but nonetheless significant anti-edematogenic effect of SRT appears between 6 and 24 hours, an effect that was strongly correlated with improvements in survival rate. The anti-edematogenic effect is observed with Exosurf® but not with Infasurf® as the surfactant replacement.
- SRT has little if any effect on the changes in epithelial lining fluid that occur within the first 4 hours after exposure. SRT does not affect the increase in alveolar epithelial-endothelial permeability marking the onset of the clinical phase of phosgene poisoning. Epithelial lining fluid IL-6 levels are greatly increased after phosgene exposure in the presence or absence of

SRT. Fibronectin levels are significantly increased after phosgene exposure in the presence or absence of SRT.

- Exosurf® appears to be largely ineffective in maintaining the alveolar surface film in vivo during the early stages of phosgene poisoning. It does not serve to normalize lung compliance nor does it prevent the massive outpouring of edema fluid that occurs within the first 6 hours after exposure. SRT with Exosurf® supplemented with Poloxamer 188 (1% w/v), a synthetic surfactant that has been shown to antagonize the inhibitory effects of fibrin on PS function, does provide a sustained salutary effect on lung compliance and terminal bronchiole patency, as well as effecting the highest survival rate observed in these studies. The salutary effects of SRT with Exosurf® supplemented with Poloxamer 188 on PS function in situ is short term.
- Exosurf® supplemented with the lipophilic antioxidant, Vitamin E, does not improve the survival rate after phosgene exposure.
- SRT appears to be highly effective in increasing survival in rats after exposure to an $LCt_{50,10,24}$ phosgene dosage and is the most effective therapy currently available for attenuating phosgene lethality.
- SRT may be accomplished by bolus instillation or by aerosolyzation. Both techniques would be applicable under battle field conditions.
- USAMRDC should continue to study SRT as a countermeasure for phosgene intoxication to determine suitability for use by US military personnel.

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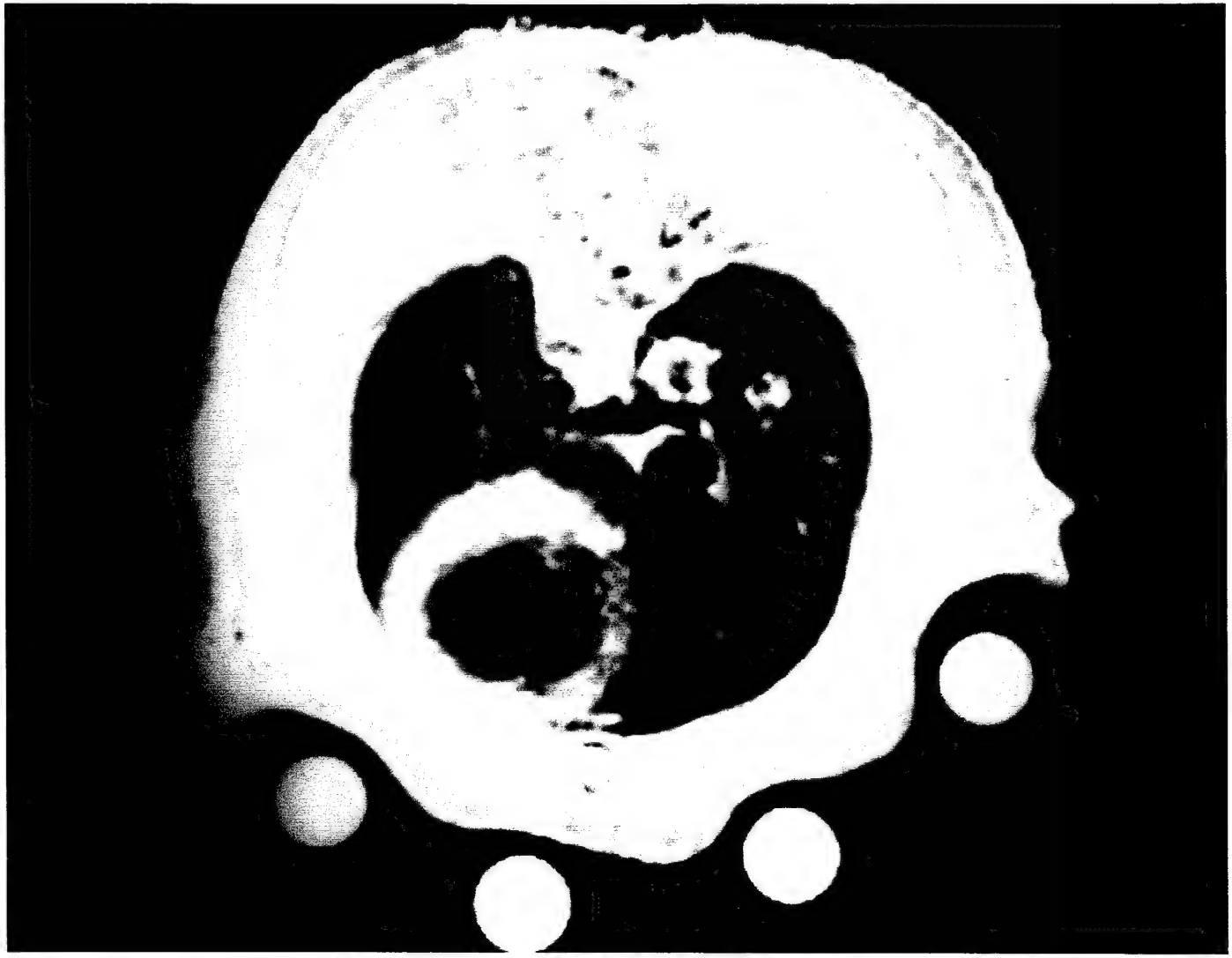
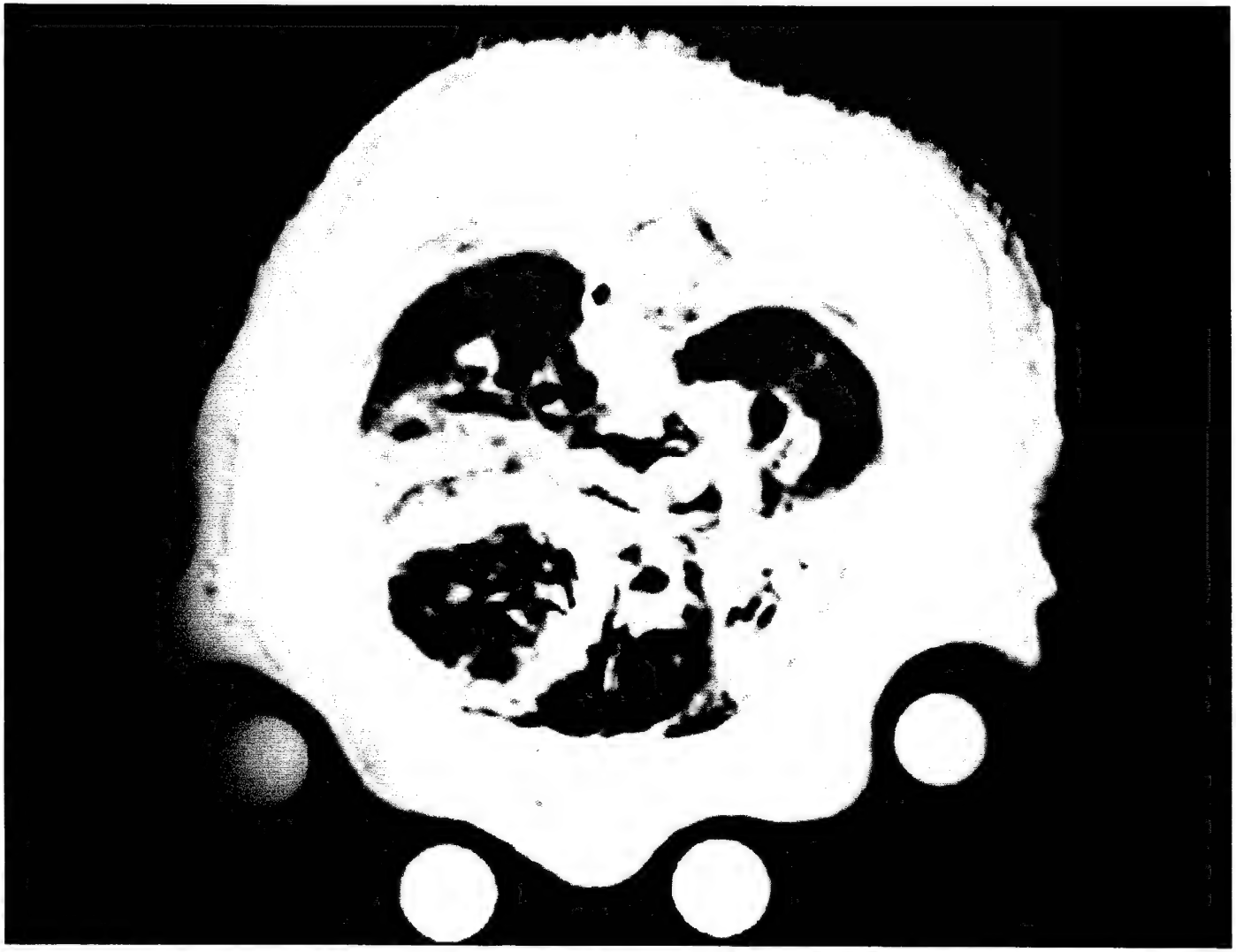


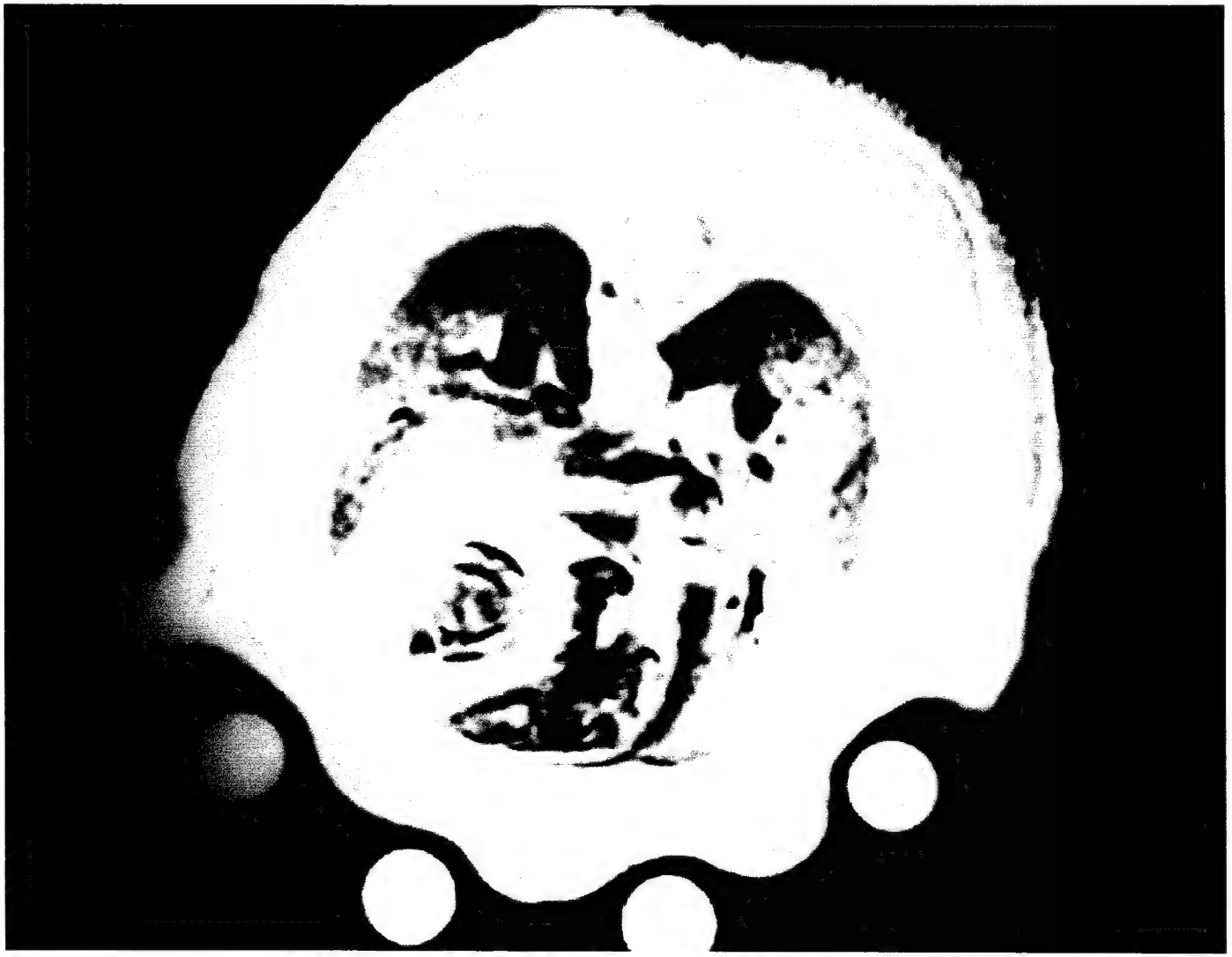
MRI transaxial section of normal rat thorax (no phosgene, no surfactant).

Untreated
Photograph 1



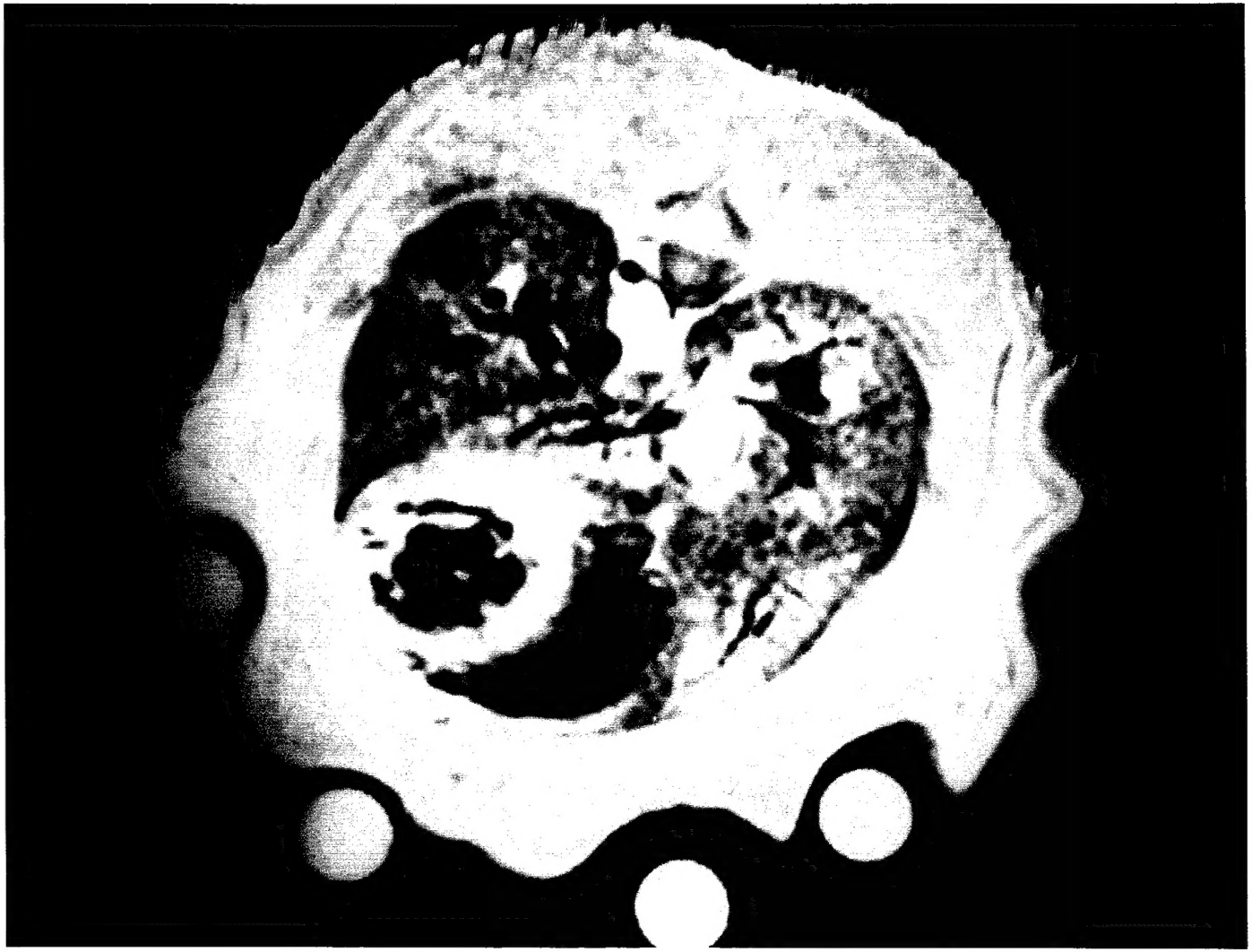
MRI transaxial section of normal rat thorax following instillation of 1 ml Exosurf®.

1 ml Exosurf
Photograph 2



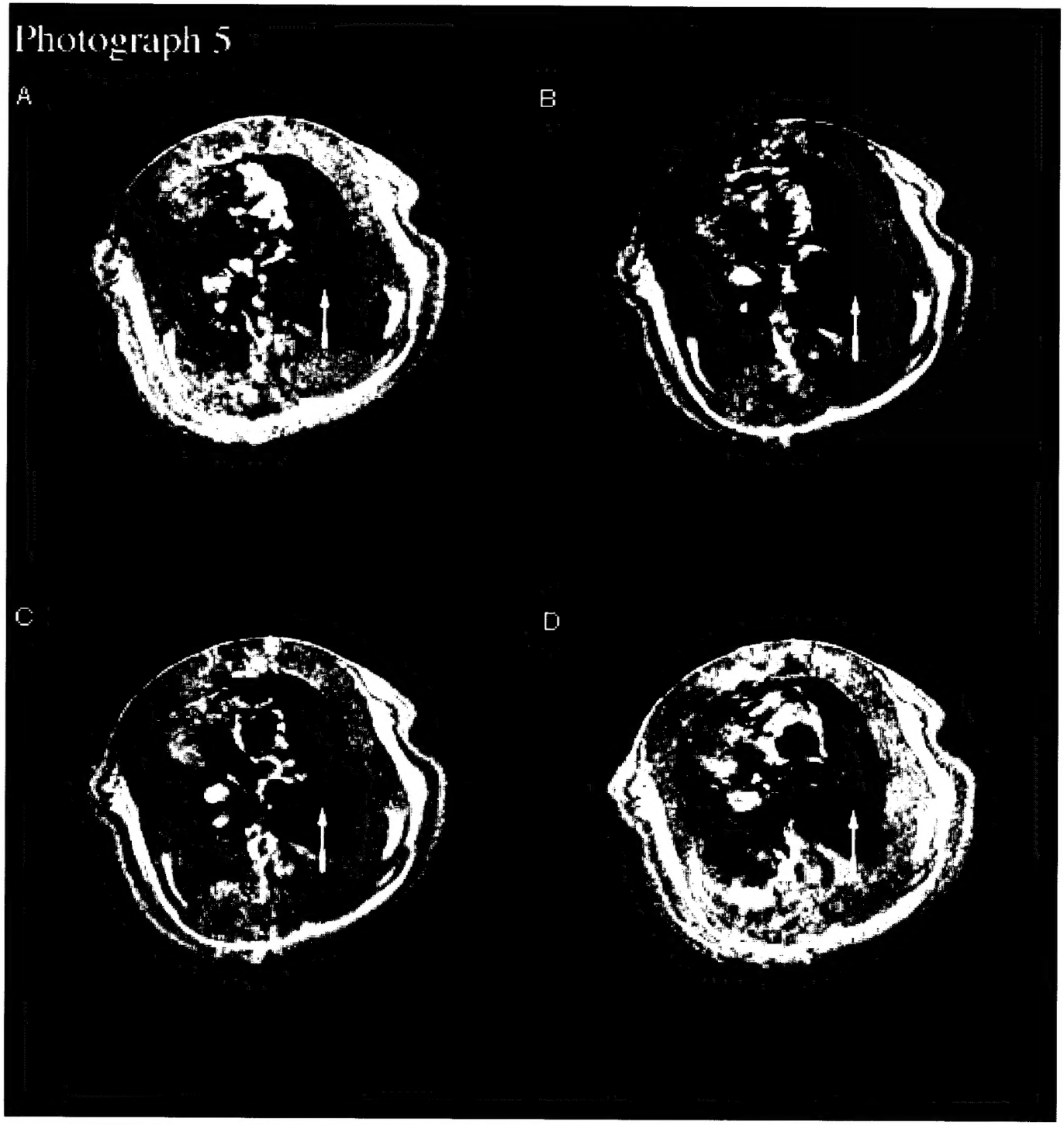
MRI transaxial section of normal rat thorax following instillation of 2 ml Exosurf®.

2 ml Exosurf
Photograph 3

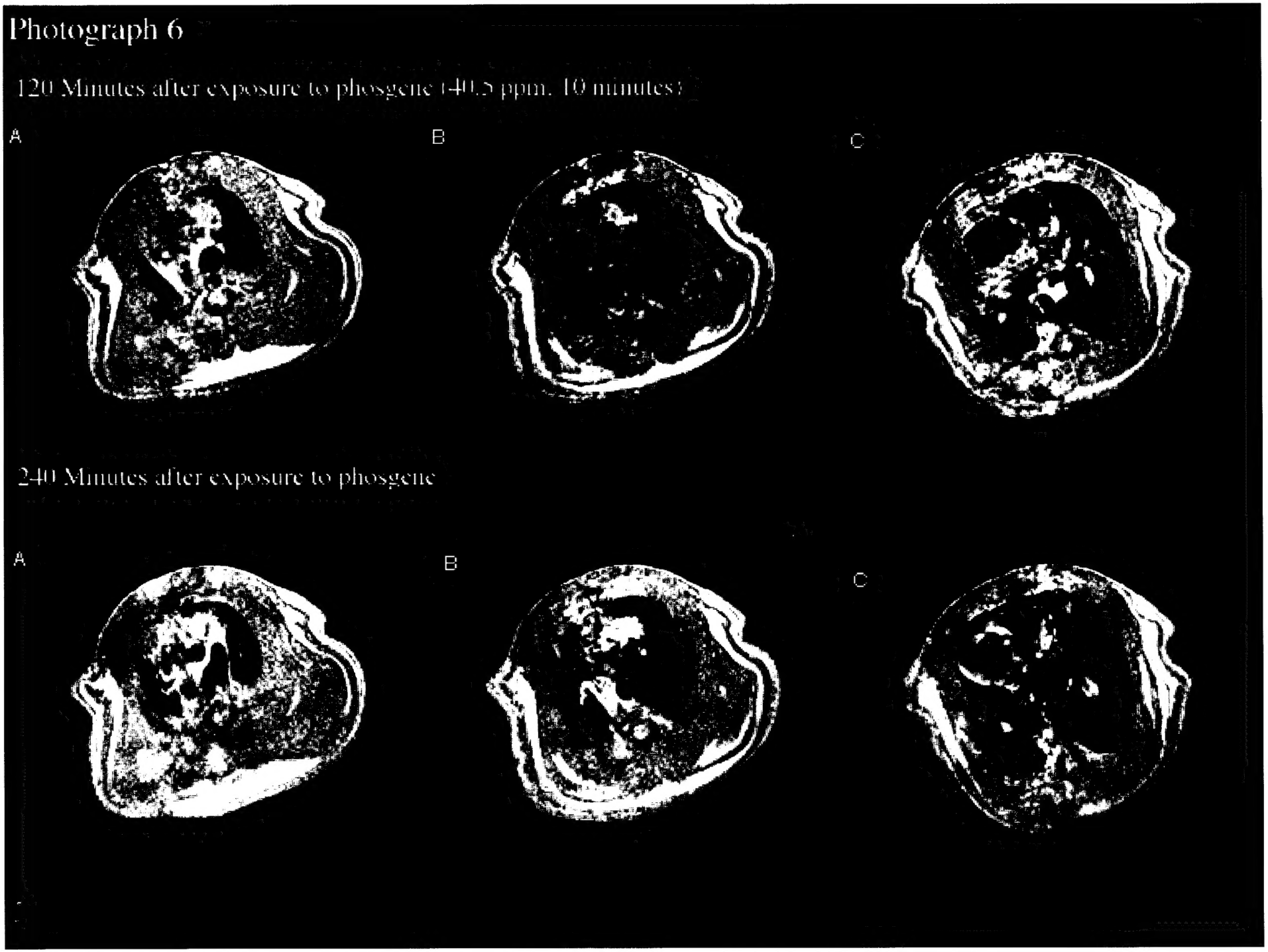


MRI transaxial section of normal rat thorax following instillation of 3 ml Exosurf®.

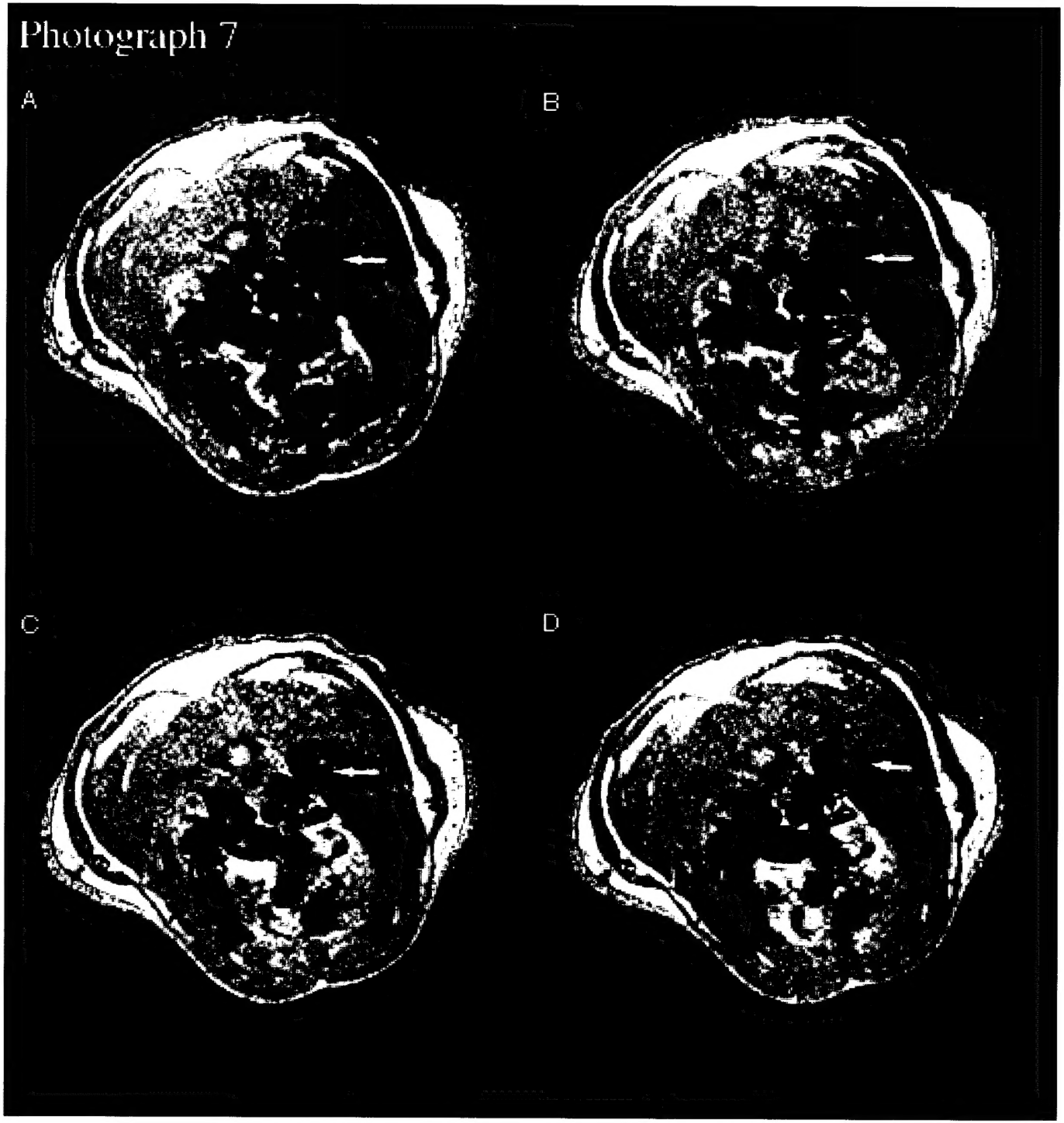
3 ml Exosurf
Photograph 4



Changes in MRI signal intensity of the lung following exposure to phosgene.



Multi-slice MRI imaging of the rat thorax following inhalation of phosgene.



Focal lesion in the lung of a phosgene exposed rat detected by MRI.